SZENT ISTVÁN UNIVERSITY
FACULTY OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES
MSC PLANT BIOTECHNOLOGY

VIRUS DIAGNOSTIC SURVEY OF GRAPEVINE ROOTSTOCK VARIETIES FROM THE STOCK COLLECTION OF PÉCS

Author:
Houhou Fakhreddine

Primary Thesis Adviser:
Professor Kiss Erzsébet

Institute/Department
Institute of Genetics and Biotechnology

Independent Consultant:
Várallyay Éva, PhD

Gödöllő
2016

TABLE OF CONTENTS

Acknowledgement

First of all, I would like to express my deepest sense of Gratitude to my Thesis Supervisor Dr.Éva Várallyay at the ABC, for her patience, motivation, enthusiasm, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my MSc Thesis.

I would like to express my very sincere gratitude to my Professor Erzsébet Kiss leader professor of the Agricultural Biotechnology group, whose expertise, understanding, and patience, added considerably to my graduate experience. I thank her for the systematic guidance and great effort she put into training me in the scientific field.

A very special thanks goes to Mrs. Carmen Iliescu research assistant in the diagnostic group of ABC center, for her inestimable help and assistance throughout the laboratory practices, and for her kindness and wonderful cooperation.

I would like to thank the committee members who will consider my Thesis dissertation.

I would also like to acknowledge the Institute of Genetics and Biotechnology for the education and support. I give my sincere thanks to all my professors from whom I get the chance to gain precious knowledge during my MSc study.

Furthermore, I am thankful to the Balassi institute for their valuable Stipendium Hungaricum Scholarship which made these research possible and for the unforgettable stay in Hungary.

I thank my classmates of the MSc Agricultural Biotechnology group, especially my wife for her encouragement and editing assistance.

Finally, and most importantly, I would like to thank, my parents, my brothers and sister as well as my uncles for their unconditional support and encouragement.

Abbreviations

AMV: Alfalfa Mosaic Virus

ArMV: Arabis Mosaic Virus

cDNA: complementary DNA

CTAB: Hexadecyltrimethylammonium-bromide

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-Linked Immuno-Sorbent Assay

EPPO: European and Mediterranean Plant protection Organization

GBLV: Grapevine Bulgarian Latent Virus

GCMV: Grapevine Chrome Mosaic Virus

GFkV: Grapevine Fleck Virus

GFLV: Grapevine Fanleaf Virus

GLD: Grapevine Leafroll Disease.

GLPV: Grapevine Line Pattern Virus

GPGV: Grapevine Pinot Gris Virus

GRLaV: Grapevine Leafroll Associated Viruses

GSyV: Grapevine Syrah Virus

GVA: Grapevine Virus A

GVB: Grapevine Virus B

ICTV: International Committee of Taxonomy of Viruses

LAMP: Loop-mediated isothermal amplification

NGS: Next Generation Sequencing

PVP: Polyvinylpyrrolidone

RsPaV: Rupestris stem pitting-associated virus

RT-PCR: Reverse Transcription Polymerase Chain Reaction

RW: Rugose Wood

TBE: Tris/Borate/EDTA

TBRV: Tomato Black Ring Virus

List of Tables

List of figures

Introduction

As a vegetative propagated plant, grapevine (Vitis spp.) is severely exposed to a broad range of viruses and viroids, estimated to 58 viruses detected worldwide, representing the largest number of viral pathogen found in a single plant Viruses affect negatively plant vigor and longevity, as well as the quality and quantity of the yields **.** Therefore, to lower the risk of the dissemination in the vineyard, efficient detection of grapevine viral infections is crucial for the preservation of healthy clones **(Martelli and Boudon-Padieu, 2006; Engel et al., 2009).**

Standardized diagnostics methods like Enzyme-linked immuno sorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) are the most common and widely approved techniques used in research laboratories for virus screening. However, these techniques have limitations which may delimit the virus detection procedure. The woody structure and anatomy of the grapevine affect the progress of diagnostics, making the extraction procedures laborious and more complex to examine compared to other plant samples (**Engel et al., 2009).**

In Hungary, Virus detection play an important role in promoting the use of virus-free stocks, which was started under the base studies of Dr. János Lehoczky and his co-workers (**Lehoczky, 1965)**. Nowadays, new viruses emerged, or might have been always present in the region but just not detected yet. New identification methods such as molecular tests (PCR) have been introduced and offer new opportunities in the diagnostic field to enhance competency and obtain a deepen knowledge on the virus-host interaction and the overall sanitary condition of the vineyards (**Cseh et al., 2012).**

The aim of our study was to present a general overview on the occurrence of some of the most prevalent grapevine viruses in a rootstock collection of Pecs in Hungary. Molecular techniques based tests RT-PCR was carried out to survey the presence of viruses and a comparative analysis was made investigating several rootstock varieties. With this method we could efficiently sample and detect virus infection in the investigated hosts.

Our results showed the presence of different viruses at the vineyard of the Research Institute for Viticulture and Oenology at Pecs. We surveyed the presence of different viruses, and have found that large number of the rootstock collection was affected.

1. Literature Review

1. Origin and History Overview

The grapevine (*Vitis vinifera* L.) is a perennial woody plant classified among the Vitaceae family, which comprehend about 60 inter-fertile wild *Vitis* species. The domestication of grapevine appear to have been initiated in between the seventh and the fourth millennia in the geographical region delimited by the Black Sea and Iran (Chataignier, 1995; McGovern et al., 1996; McGovern and Rudolph, 1996; Zohary, 1996; Zohary and Hopf, 2000) From the diverse *Vitis* species, *V.vinfera* was the only one which have been of an economic interest to humans (Hardie, 2000; Mullins et al., 1992; Zohary and Hopf, 2001). However, other *Vitis* species like the North American V. rupestris, V. riparia or V. berlandieri, have valuable breeding benefits as rootstock because of their acquired resistance against some grapevine pathogens, such as Phylloxera, Oidium and mildews. (Rossetto et al., 2002; Sefc et al., 2003; Crespan, 2004; This et al., 2004).

Two subspecies of *V.vinifera* co-exist in Eurasia and North Africa which have been historically separated in regards to their morphological differences: the cultivated subsp. *vinifera* (or *sativa*) and the wild *subsp. silvestris* (or *sylvestris)* (Zohary, 1995).

2. Morphology and anatomy

As described by Keller (2010) "Grapevines are very vigorous, woody climbers named lianas that are perennial (i.e., they live more than 2 years), polycarpic (i.e., they flower many times during their life), and deciduous (i.e., they shed their leaves each year)".

The vine's lifespan may be extend when it is propagated vegetatively by mean of artificial cutting bud grafting, and tissue culturing, where rooted cutting an grafted plants displayed a faster regeneration when compared to mother plant. (Munné-Bosch, 2008). For instance, leaf gas-exchange rates, and fruiting of the propagated vines are independent of the age of their original propagated materials where they were taken from (Keller, 2010).

As a general rule in higher plants, grapevines structure is composed of two main parts: a vegetative part including roots, trunk, shoots, leaves, and tendrils, where the aboveground section (trunk and shoots) is named the vine's canopy, and a reproductive part consisting of clusters with flowers or berries. (Keller, 2010).

3. Genetic variation in grapevine

According to Franks, et al. (2002) three processes have had a significant impact on the development of cultivated grapevines:

Sexual reproduction: The production of new genotypes is mainly conducted by one of these methods, either by crossing different varieties or by self-fertilization, where the main result is to produce a new genetic combination of the parental alleles with high heterozygosity, contributing to phenotypic variations and segregation of traits in the progeny population.

Vegetative propagation: (asexual) Nowadays, the vegetative propagation is the principal method of V. vinifera reproduction, where the cutting method is used to preserve highly desirable genotypes for the sake of homogenous plantations originated from one cultivar. For its convenience it is also preferable in transporting cultivars from one region to another.

Somatic mutations: The main purpose of clonal propagation is to maintain a homogenous genotype in all plants, however, somatic mutation may occur in some cuttings which might lead to a disparity in the genotype of the same cultivar, in some case resulting in different phenotype, referred to as clonal variation.

The grapevine attains a great interest over the past five years in the matter of genomic research due to its small diploid genome size of 475–500 Mb in contrast to other plants (it is approximately four times the size of *Arabidopsis* and one-sixth the size of the corn genome) (Thomas, et al. 1993; Lodhi and Reisch, 1995), furthermore the grape varieties are heterozygous genotypes and practically all modern cultivated varieties are hermaphroditic, self-fertile and out-cross easily (This et al., 2006).

4. Grapevine rootstock Varieties

Rootstock is defined by Robinson (2015) as "the root system of a grapevine to which a fruiting variety, or scion, is grafted. In most vineyards in the world, European wine-producing *vinifera* vines are grafted on rootstock which are, with few exceptions, either varieties of one American Vine species or more commonly hybrids of several".

The main reason behind using rootstock is to overcome different pest or diseases, and some particular soil condition which drawback the plant productivity

a. Rootstock characteristics

Chien (2008) described the main characteristics that a rootstock may offer by one or more of the following assets:

• Protection against soil-borne pests and diseases such as phylloxera and nematodes

• Tolerance to environmental conditions such as drought, wet soils, salinity or lime

• Influence on vine vigor and size

• Influence on nutrient and water availability

• Effect on vegetative cycle and fruit ripening

• Ability to propagate

b. Rootstock species

According to Goldammer (2015) most rootstocks are either native North American species or hybrids of two or more of these species, including *Vitis riparia, Vitis berlandieri*, and *Vitis rupestris*.

- ***V. riparia x V. rupestris***

Rootstocks derived from these crosses prefer deep, fertile and moist soils. These rootstocks offer low-moderate vigor to the scion and in certain situations hasten ripening (Goldammer, 2015).

- ***V. berlandieri x V. rupestris***

This interspecific hybridization created by Franz Richter in 1902 from which derived the varieties:

• 110 Richter

• 99 Richter

These rootstocks offer moderate-high vigor to the scion, are drought tolerant, and better adapted to warm to hot regions. These rootstocks are suited to a wide range of soil types of low to moderate depth and fertility (Goldammer, 2015).

- **V. berlandieri *x* V. riparia**

Berlandieri *x* Riparia hybrids are the most important grape rootstocks in use presently (Galet, 1988). They are Issued from various crossing and consecutive selection to acquire a combination of important characteristics as resistance to phylloxera and lime tolerance as well as other useful traits (Becker, 1968).

The main popular clones resulting from the crossing Berlandieri **x** Riparia are:

- Teleki 8 B
- Kober 5 BB
- Kober 125 AA
- Teleki 5 C / 5 C Geisenheim
- Selektion Oppenheim 4 / SO 4
- Binova

These rootstocks offer moderate-high vigor to the scion depending on the soil type. However, rootstocks in this group are more vigorous than those from V. riparia *x* rupestris crosses especially under available precipitation (Goldammer, 2015).

5. Grapevine situation in Hungary

At the beginning of rootstock breeding, it was the French and later the Italian experts, who led the way. However, within a short period of time. Hungarian rootstock breeders gained high respect among them (Reynolds, 2015).

One of the pioneer of Hungarian breeding vine history was Zsigmond Teleki (1854-1910) who realized the importance of rootstock breeding after the destruction caused by phylloxera in wine regions across Europe which had caused serious economic damage (Reynolds, 2015).

Several rootstock breeders used the propagation method of his rootstock hybrids for their own experiments. Among them were Fuhr (Oppenheim/Germany), Birk (Geisenheim/Gremany) and Kober (Klosterneuburg/Austria) (Hegedus et al., 1966).

The main valuable rootstock varieties that Teleki developed were ; Teleki-Fuhur SO4, Teleki-Kober 5BB, Teleki 5C, Teleki-Kober 125 AA, Teleki 8B and Teleki 10A (Csepregi and Zilai, 1955).

According to Lehoczky and Reichart (1968), Lehoczky and Tasnády (1971) the detection of virus and virus-like diseases of grapevine in Hungary was started in late1960's and the regular virological screening was introduced in 1972. Until now 17 virus and virus-like diseases have been detected (Lázár 1996, Kölber et al. 1997, Lázár et al. 2002, Lázár & Bisztray 2011).

6. Grapevine Viruses Diseases

Considering the Grapevine being a perennial plant it is, therefore, most vulnerable to be the sink for more than 60 virus and viroids from a broad range of families and genera.

We can broadly divide the viruses infecting grapevine into two groups according to their economic importance and geographical distribution in the world.

1. Major diseases: (also called traditional diseases) that are the cause of great economical loss and responsible of significant decline in productivity of the vineyards. In that order we can mention grapevine leaf roll disease (GLD), rugose wood (RW) complex, and nematode-borne viruses which are considered as the most destructive and wide spread virus disease in viticulture (Rayapati et al, 2008).

2. Minor diseases: their economic significance is secondary with and/or a limited geographical distribution. It has been reported that the larger part of virus diseases belong to this latter category, nevertheless all viruses, regardless of their economic importance, should be taken in consideration in favor of sanitary regulations (Rayapati et al, 2008).

As categorized by (Cseh et al., 2008) Grapevine viruses or virus like has been represented on the basis of most characteristic symptoms as:

a) Degeneration,
b) Leaf roll,
c) Fleck,
d) Rugose wood,
e) yellow mottle,
f) Line pattern,
g) Enation**,**

6.1. Grapevine degeneration

Fanleaf degeneration/decline is a severe wide spread disease which was first detected in Hungary by Sárospataki in 1964. It is caused by several types of Nepoviruses: Grapevine fanleaf virus (GFLV), Arabis mosaic virus (ArMV), Grapevine chrome mosaic virus (GCMV), Grapevine Bulgarian latent virus (GBLV) and Tomato black ring virus (TBRV) (Cseh et al., 2008).

Grapevine fanleaf virus (GFLV) is by far the most epidemic infection in vineyards. It belongs to the genus Nepovirus of the family Comoviridae (Mayo and Robinson, 1996), where vines display distinguishable symptoms of the disease described by Martelli (1993) as followed:

(1) Fanleaf deformation: Leaves are variously and severely distorted, asymmetrical, cupped and puckered and exhibit acute dentations

(2) Yellow mosaic: Affected vines show chrome-yellow discolorations that develop early in the spring and may affect all vegetative parts of the vines (leaves, herbaceous shoot axes, tendrils and inflorescences)

(3) Vein banding: Bright yellow bands may develop along the major veins

Some lethal symptoms can cause reduction in yield (up to 80%) and quality deterioration of the affected vines, which leads to a shortened productive lifespan of the vineyards that generally ends in vine death (Oliver and Fuchs, 2011).

Field symptoms are usually sufficient for the detection of (GFLV) infected vines, whereas, a more sensitive Vitis species can be used as an indicator plants transmitted by grafting (Martelli, 1993; Cseh et al., 2012).

6.2. Leafroll disease

Grapevine leafroll associated viruses (GRLaV) are members of the family Closteroviridae and belong to the genera: Ampleovirus and Closterovirus. The disease has emerged in all major grape-growing regions of the world, effecting adversely the productivity and quality of both wine and table grapes. We can distinguish two main symptoms of the infection taking place in autumn season, strong red leaf color appear in dark-fruited varieties, While, In lighter fruited varieties, a general chlorosis will develop (Golino et al, 2002).

Virologists have identified 9 grapevine leafroll-associated viruses (GLRaVs) in wine grapes showing GLD symptoms. They exclusively attack the vascular tissue (phloem) of the vines engendering a lower yield (up to 30-50%), fewer clusters and delayed fruit ripening (Martinson et al, 2008).

GRLaV was firstly reported by Lehoczky and co-workers in 1969. Considering the speculation of Lázár et al. (1995) "GLRaV 1-4 have been found in Hungary". As well as GLRaV- 7 (Choueiri et al., 1996).

Table 1: Classification and some properties of Grapevine leafroll-associated viruses (GLRaVs). Adapted from "Taxonomic revision of the family Closteroviridae", by, Martelli et al. Journal of Plant Pathology, Vol 94 (1), 8. (2012) by Edizioni ETS Pisa.

Virus	**Genus**	**Coat protein (kDa)**	**Genome size (nts) (GenBank Accession No.)**	**Vectors**	**First record Boscia et al. (1995) and this paper**
GLRaV-1	Ampelovirus	34	18,659 (JQ023131)	Mealybugs, soft scale and scale insects	Gugerli et al. (1984)
GLRaV-2	Closterovirus	22	16,494 (AY88162)	Unknown	Zimmermann et al. (1990)
GLRaV-3	Ampelovirus	35	18,498 (EU259806)	Mealybugs, soft scale and scale insects	Zee et al. (1987)
GLRaV-4	Ampelovirus	35	13,830 (FJ467503)	Mealybugs	Hu et al. (1990)
GLRaV-5	Ampelovirus	35	13,384[a] (FR822696)	Mealybugs	Zimmermann et al. (1990); Walter and Zimmermann (1991)
GLRaV-6	Ampelovirus	35	13,807 (FJ467504)	Mealybugs	Gugerli and Ramel (1993); Gugerli et al. (1997
GLRaV-7	Unassigned in the family	37	16,496 (HE588185)	Unknown	Choueiri et al. (1996)
GLRaV 8[b]	Ampelovirus	37	ND	Unknown	Monis (2000)
GLRaV-9	Ampelovirus	35	12,588[a] (AY29781)	Mealybugs	Alkowni et al. (2004)

a)nearly complete sequence; b) Cancelled from the 9th ICTV Report (Martelli et al., 2011a); ND) not determined.

Dissemination of Leafroll-associated virus is generally determined by two major mediums, the most common factor is carried out through vegetative propagation and grafting where the infection can spread over long distances by the dint of the propagation and planting materials

used; additionally, two insect vectors, namely, mealybugs and soft scales, demonstrate their ability to transmit GLARaVs between vines and occasionally, between neighboring vineyards (Cieniewicz and Fuchs, 2015; Jordan, 2013).

6.3. Grapevine chrome mosaic virus (GCMV)

Grapevine chrome mosaic virus (GCMV) is a Nepovirus that derive from the Secoviridae family. It was originally isolated from diseased Hungarian vines near the Lake Balaton by Martelli, Lehoczky and their co-workers (Martelli, 1966; Lehoczky et al., 1984).

We can recognize the infection by the chrome-yellow to whitish discolorations of the leaves, a visible lack of vigor, and unfruitfulness of the vines. Malformations and chloric mottling of the leaves could be identify in some strains whereas in other remain symptomless.

Soil-borne is the presumed virus responsible for the dissemination of the disease**;** specifying that the nematode Xiphinema vuittenezi is the one associated with the spread of the disease in Hungary (Martelli & Sàrospataki, 1969).

6.4. Arabis mosaic virus (ArMV)

According to the European and Mediterranean Plant protection Organization (EPPO) Arabis mosaic virus (ArMV) has a wide host range including principally Vitis spp. It belong to Nepovirus genus from the family Secoviridae. Closely related to grapevine fanleaf virus (GVFLV) **(**Cseh et al., 2008). The disease was initially spotted in Hungary in 1966 by Martelli and Lehoczky (Martelli and Boudon-Padieu, 2006). And is mainly transmitted by the soil-inhibiting nematode Xiphinema diversicaudatum (Jha & Posnette, 1959; Harrison & Cadman, 1959).

The most common symptoms induced by ArMV are leaf mottling and flecking, stunting and several forms of deformation including enations (EPPO, N.D).

6.5. Grapevine fleck virus GFkV

Fajardo et al (2012) describe Grapevine fleck as “graft-transmissible disease of grapevine, caused by Grapevine fleck virus (GFkV), a phloem-limited and non-mechanically transmissible virus”.

In Hungary Fleck disease was first found in 1981 by Lehoczky (Lehoczky and Farkas, 1981) (Walter and Cornue, 1993; Sabandzovis et al., 2001). (GFkV) is predominantly latent in V. vinifera cultivars, except in its indicator host V. rupestris where it shows specific foliar symptoms: clearing of veinlets and translucent spots, wrinkling and upward curling of the leaves. (Walter and Cornue, 1993; Sabandzovis et al, 2001).

As a general rule, transmission of grapevine viruses is mostly done through non-sterile planting material, but could also be conducted via insect vectors including mealybugs and scale, nematodes and aphids

6.6. Rugose Wood Complex

Rugose wood is a complex disease that affect Vitis species, it is characterized by modifications of the woody cylinder (Martelli et al 2007)and is responsible for graft incompatibility, delayed budburst, severe decline and even death of vines (Cseh et al., 2008). In Hungary it was identified by Martelli et al. (1967). In Hungary it was detected by Martelli et al. (1967).

Four different disorders can be recognized by biological indexing where only three of them have been associated with viral infection:

1. Rupestris stem pitting; caused by the "Rupestris stem pitting associated virus" (RSPaV) (Zhang et al., 1998). The virus is not mechanical transmissible and no natural vector has found until now (Nakaune *et al*., 2008).
2. Kober stem grooving; grapevine virus A (GVA) (Garau et al., 1994). It is a Phloem-limited virus which can be transmitted by mealybug (Minafra et al., 1997)
3. Corky bark; caused by grapevine virus B (GVB) (Bonavia et al., 1996) which is transmitted by mealybugs (Martelli and Boudon-Padieu, 2006)
4. LN 33 stem grooving; no virus has been found associated with this syndrome.

In 2009, the International Committee on Taxonomy of Viruses (ICTV) modified the list and the classification of the viruses and added a new family *Betaflexiviridae* (Carstens, 2010) which includes the rugose wood complex caused viruses GVA, GVB and GRSPaV.

Various symptoms can be patent depending on the vine cultivars which make individual diseases difficult to distinguish. Active diagnostic testing is required to confirm the presence of viruses. (Constable and Rodoni, 2011) Generally, diseased vines tend be dwarfed and less vigorous than normal and might show a delay in bud break during spring time (Jordan, 2014).

Propagation of infected plant material seems to be the primary mechanism of spread for the viruses GVA, GVB, and RSPaV through Graft transmission from rootstock to scion and vice versa (Constable and Rodoni, 2011; Jordan, 2014).

6.7. Grapevine yellow mottle

The causal agent of yellow mottle is alfalfa mosaic virus (AMV). It was detected in Hungary by Lehoczky and Beczner (1980). AMV is a polyphagous virus infecting a great number of plant species in nature and artificially (Hull, 1969). The affected vines show various patterns of yellow discolorations that does not extend to the veins which remain green, whereas the proliferation of the disease is conducted by grafting, use of contaminated propagation materials, and aphids (Martelli, 1993).

6.8. Grapevine line pattern

Line pattern symptom caused by Grapevine line pattern virus (GLPV) which is considered to be a putative member of the genus Ilarvirus in the family Bromoviridae. This disease has been reported only from Hungary, where it was firstly described in the world by Lehoczky et al. (1987). Field symptoms shows a bright yellow discolorations of the leaves forming marginal rings of variable size, scattered spots or blotches or maple-leaf line pattern(Martelli, 1993). The transmission path is carried out through bud wood and seeds (Lehoczky, Martelli and Lazar, 1992).

6.9. Grapevine Pinot Gris Virus (GPGV)

Grapevine Pinot gris virus (GPGV) is classified among the genus Trichovirus of Betaflexiviridae family. It was first discovered in 2012 in Italy from Vitis vinifera 'Pinot gris' exhibiting leaf mottling and deformation (Giampetruzzi et al. 2012). However, the pathogenicity of GPGV remains to be clarified, as it is not consistently associated with symptomatic plants (Beuve et al., 2015).

6.10. Grapevine Syrah Virus-1 (GSyV-1)

Grapevine Syrah virus-1 is a member of the Marafivirus genus within the Tymoviridae family. (Monis, 2009)This virus was independently reported from cultivated and wild grapevines (Martin et al., 2013).

As for the symptoms, affected vines displayed swollen graft unions, cracking and pitting of the wood, stem necrosis, leaf reddening and scorching, vine decline, and death of the vines (Al Rwahnih et al., 2009)

7. Virus Diagnostics methods

To prevent grapevine pathogens including viruses, viroids, phytoplasma, bacteria and fungi from spreading in the vineyards and infecting the stock plantations, several diagnostic approaches have been developed aiming to obtain pathogen-free plants which cover biological, serological and molecular assays for a better detection and elimination of the different pathogens (Bisztray et al. 2012, Szegedi et al. 2012).

a. Standard diagnostic methods

The most well-known and widely used detection method of grapevine viruses are ELISA and RT-PCR (reverse transcription-polymerase chain reaction) approaches (Monis, 2011; 2012).

i. Detection by ELISA (enzyme-linked immuno-sorbent assay)

Clark and Adams (1977) published the first enzyme-linked immunosorbent assay (ELISA) method for the detection of Plum pox virus and Arabis mosaic virus (ArMV), and was considered as a breakthrough method back then, and opened new perspectives to the modern phytodiagnostics field (Boonham et al., 2014).

ELISA detection approach rely on binding antibodies with the protein of the virus of interest on a test plate. Provoking an enzymatic reaction that will develop a coloration if the sample turns to be positive (Monis, 2011; 2012).

The serological essay ELISA was described by Boonham et al (2014) as **"**most versatile assay for simple and sensitive virus testing" with a broad virus detection capability, nonetheless, the assay have shown some limitations when detecting virus present at low concentration in a vine sample which could be imputable to seasonality (Monis, 2012).

ii. Detection by Polymerase Chain Reaction methods (PCR)

The first publication released on PCR methods for virus detection date to the early 1990s (Vunsh et al., 1990). Nucleic acid-based methods like PCR have demonstrated an exquisite level of specificity and sensitivity to a broad range of virus and viroids, where, real-time PCR and RT-PCR assays showed the most reliable and reproducible results.

The main advantage of using PCR-based methods is their sensitivity in detecting low concentration of the virus in the plant sample, but are unfortunately not qualified to detect viral variants (Monis, 2011).

b. Novel molecular diagnostic methods

i. Loop-mediated isothermal amplification (LAMP)

Isothermal amplification is an efficient and cost-effective alternative to PCR which does not require thermocycler apparatus **(**Capote et al., 2012). Loop-Mediated Isothermal Amplification (LAMP) was initiated by *Notomi* and coworkers (2000) using six oligonucleotides primers (internal, external and loop primers), to generate an amplification product which contains single-stranded loop regions to which primers can bind without thermal DNA denaturation required (Notomi et al., 2000). This technology can be used for the amplification of RNA templates (RNA viruses, viroids) in presence of reverse transcriptase (Czotter et al; 2015), An RT-LAMP based method was developed by Walsh and Pietersen (2013) for GLRaV-3 and proved to be as sensitive as nested PCR."

ii. Micro and macro array techniques

DNA micro- and macro-arrays are generally used for gene expression profiling but are also powerful tools for identification and differentiation of plant pathogens (Anderson N. et al., 2006; Lievens & Thomma, 2005). The DNA array technology allow a specific and precise SNP detection; which is a valuable feature for diagnostic application where differentiation of pathogens related species may depend only on a single base pair polymorphism for a target gene (Capote et al., 2012), moreover, a former PCR amplification prior to hybridization is necessary to compensate the lack of sensitivity of the array technique (Capote et al., 2012).

iii. DNA barcoding technique

Barcoding is a taxonomic method that combines PCR amplification and sequencing, by amplifying a short genetic marker specifically designed for the target borders of the DNA to be identified an organism as belonging to a particular species (Czotter et al; 2015; Capote et al., 2012).

iv. Deep (Next generation) sequencing techniques

Next-generation (high throughput, deep) sequencing (NGS) had remarkably advanced the previous sequencing techniques by using standard dye-terminator methods (Barba et al., 2014) By searching for all the expressed RNA of the host plant including pathogenic RNA, employing different sequencing platforms such as (Roche 454 and Illumina) (Boonham et al., 2014; Czotter et al; 2015).

NGS techniques led their ways in metagenomics based strategy as a powerful tool for identification of quantification of novel viruses in one step (Dunowska et al., 2012; Prabha et al., 2013). The technique had been put in practice and successfully indicates the existing viruses in a grapevine plant presenting characteristic symptoms of Syrah disease (Al Rwahnih et al., 2009). Ultimately, deep sequencing technologies permit the identification of plant virus without prior information on the virus macromolecular sequence, which make multiple viruses sequence investigation possible through the use of non-sequence specific primers (Adams et al., 2009; Kreuze et al., 2009).

Both Donaire et al. (2009) as well as Kreuze et al. (2009) emphasized the important role of RNA interference in the defense mechanism of the plant, by generating small RNA libraries of infected plants using deep sequencing as a diagnostic tool that will promote virus identification.

2. Materials and Methods

For the purpose of the virus diagnostic survey, a selection of rootstock was necessary to limit the analyzes to the most important rootstock varieties of Grapevine in one of the main vinicultural region of Hungary (Pécs), where 34 rootstock samples were collected, and examined for the presence of the major grapevine viruses which are listed in Table 2. RNA was extracted and used as a template for the cDNA synthesis, for generating cDNA library (pools) that will facilitate the detection of viruses using virus-specific primers especially designed to anneal to the target sequence that will be patterned at the end by gel electrophoresis.

1. Sample collection and origin

The rootstock collection were obtained from the Research Institute of Viticulture and Enology of Pécs on the date of 27 July of 2015, where various typical varieties were selected. Samples were collected from different plant organs: shoot tip, old leaf, young leaf, flower, and tendril of each rootstock variety.

Table 2: Name of the different grapevine rootstock variety at the collection of Pécs.

n°	Rootstock variety name
1.	Teleki 8B
2.	Teleki 5C I
3.	Teleki-Kober 5BB
4.	Szilagyi 157 Pécs
5.	Riparia portalis
6.	Rupestris du Lot
7.	Rupesris metallica
8.	Chasselas x Berlandieri 41 B M. et de G
9.	Aramon x Rupestris G.1
10.	Aramon x Riparia 143 B M. et de G.
11.	Mourvédre x Rupestris 1202 C.
12.	Rupestris x Berandieri T .10A
13.	Solonis x Riparia 1616 C
14.	Golia
15.	Galiardo
16.	Riparia x Rupestris 101 – 14 M et de G.
17.	Riparia Martin de Perrier

n°	Rootstock variety name
18.	Teleki-Fuhr S. O.4
19.	Teleki 5C Gm. 6
20.	Teleki 5C Gm. 10
21.	Teleki-Kober 5BB Gm. 13
22.	Teleki-Kober 5BB Wei.48
23.	Teleki 5C wed.
24.	Teleki-Kober 5BB Fr. 148
25.	Teleki-Kober 5 BB
26.	Teleki 5C II
27.	Teleki 5C P
28.	Teleki-Kober 5 BB P XII.4
29.	Teleki-Kober 5 BB P XVIII.37
30.	Teleki-Kober 5BB Cr 2.
31.	Borner
32.	Fercal
33.	Richter 110
34.	Richter 140

2. RNA extraction

To isolate RNA was extracted from different parts of the collected sample, by using Cetyltrimethylammonium bromide (CTAB)-based protocol as follows:

The extraction buffer consisted of: 2% CTAB (hexadecyltrimethylammonium-bromide), 2.5% PVP (polyvinylpyrrolidone), 100 mM Tris base with a pH of 8.0, 100, 25 mM EDTA and 2 M NaCl. These components were heated at 65°C in water bath. A quantity of 850 µl extraction buffer was measured in each labelled 2 ml Eppendorf tubes, then placed into the 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 preheated extraction buffer and 17 µl ß- mercaptoethanol, the mixture was put back to the Eppendorf tubes and vortexed thoroughly. Next, samples were incubated at 65°C in water bath for 10 minutes, and vortexed at least once. Then, 850 µl chloroform-isoamyl alcohol was added to the samples followed by tube inversion for few times. The tubes were centrifuged at 10.000 rpm for 10 minutes at 4°C, Then, the supernatant (upper phase) of the solution was transferred into new labelled tubes which already contained 800 µl chloroform-isoamyl alcohol and centrifuged at 10.000 rpm for 10 minutes at 4°C New 1, 5 ml Eppendorf tubes were labelled in which 250 µl 9 M LiCl were measured, after centrifugation, supernatants were transferred into these new tubes followed by a quick invertion of the tubes. Subsequently, tubes were incubated on ice for 30 minutes. Samples were centrifuged again at 13.000 rpm for 20 minutes at 4°C. Supernatant part of the solution was discarded, the pellet was resuspended in 450 µl SSTE preheated to 65°C. SSTE was composed of (1 M NaCl, 0.5% SDS, 10 mM Tris base at 8.0 pH and 1 mM EDTA), followed by vortexing and 450µl chloroform-isoamyl alcohol was added to the solution. It was inverted briefly, centrifuged at 10.000 rpm for 10 minutes at 4°C. While new 15 ml Eppendorf tubes were labelled in which 280 µl izopropanol and 30 µl 4 M Na acetate was measured. The supernatant of the solution was transferred to them, centrifuged at 13.000 rpm for 20 minutes at 4°C. The supernatant part were 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 and then, by removing the supernatant, dried for 10 minutes in speed vac. The pellet was resuspended in 25 µl sterile water and vortexed gently.

Examination of the quantity and quality of the extracted RNA was done by gel electrophoresis where RNAs were detected by 1.2% agarose gel electrophoresis in TE buffer, stained with ethidium bromide and visualized under UV light. To do this 3 µl of the extracted RNA were mixed with 5 µl FDE loading dye and 2 µl sterile water, then denatured at 65°C for 5 min to eventually run 10 µl from each RNA sample on 1.2% agarose gel.

On the other hand, the concentration of the extracted RNA in each sample was determined using NanoDrop spectrophotometer.

3. cDNA synthesis

3.1. Gene pool creation

By bulking the extracted RNA of the different organs (old leaf, young leaf, flower and tendril) of a single rootstock variety, 34 RNA pools were generated, creating a mixture of RNAs with different concentration, considering that each plant part contained different concentration of RNA, e.g. the concentration of RNA found in old leaves or the tendrils was much lower compared to younger leaves or the flowers, which is justified by the age of the organs and their cellular activity in general. In consequence of this, a selection of the best samples was settled to ensure accurate results for the preparation of the RNA pools; mainly young leaves and flowers were selected for further analysis due to their high RNA content. Afterwards, each RNA pool was collected in an Eppendorf tube and stored on ice. A NanoDrop measurement of the RNA pools was undertaken after centrifuging and vortexing the different RNA mixes.

3.2. cDNA synthesis protocol

The first strand cDNA synthesis was realized using the "RevertAid™ First Strand cDNA Synthesis Kit" which is a complete system for efficient synthesis of first strand cDNA from mRNA or total RNA templates. Starting by adding 0.25 µl of Random Hexamer Primer which do not require the presence of the poly (A) tail, therefore, they can be used for transcription of the 5'-end regions of mRNA or cDNA synthesis of RNA species lacking a poly (A) tail (e.g., microRNAs), continuously adding 0.5 µg of template RNA and water until reaching 3.12 µl as a total volume to each sterile tube. It is required to let the mix chill on ice, and spin down. Incubation must be done at 65°C for 5 min, then repeating the chilling and spinning down process.

A reaction mixture was added to the previous RNA mix incorporating 1 µl of 5x Reaction buffer, 0.5 µl of 10 mM dNTP, 0.13 µl Ribolock RNase inhibitor (which protects RNA templates from degradation), 0.25 µl Revertaid reverse transcription enzyme (RT), (a recombinant M-MuLV RT which maintains activity at 42-50°C and is suitable for synthesis of cDNA up to 13 kb), at the end the total volume was at 5 µl, to homogenize the reaction mixture gentle mix and brief centrifugation was performed before the incubation of the mixture was started and carried out as follows in Table 3:

Table 3: Incubation program followed for cDNA synthesis protocol

Procedure	Temperature °C	Time
Incubation	25	10 min
	42	50 min
	45	10 min
	70	5 min

The resulted cDNA was stored at -20 ºC.

4. Quality check of the cDNA

The cDNA produced by using random primer derives not only from viral RNA but also plant host mRNAs. A quality test of the reaction was performed by amplifying a part of the host actin mRNA 'actin test', using the "Phire Green Hot start II DNA polymerase". This was started by diluting an aliquot of the cDNA generated by 10x, then proceeded to a gentle vortex and brief centrifugation of all PCR reagents after thawing. The preparation of the reaction mixture consisted of the different components described in the following table (Table 4):

Table 4: Quantity of the different reaction components of the cDNA control PCR mixture

Component	Quantity (in each tube)
Water	6.1 µl
5x Phire Green Reaction Buffer	2 µl
Primer A (Vv actin 601 s)	0.5 µl
Primer B (Vv actin 1200 as)	0.5 µl
10 mM dNTP	0.2 µl
Phire Hot Start DNA Polymerase	0.2 µl
Template 10x RT	0.5 µl
Total Volume	10 µl

The PCR program was performed in a thermal cycler according to the following steps (Table 5):

Table 5: 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	∞	-

For visualizing the cDNA product, gel electrophoresis was carried out on 1.2% agarose gel. A distinct 599 bp PCR product should be visible to confirm the validity of the cDNA synthesis.

5. Virus diagnostics by PCR

5.1. Virus detection protocol and PCR amplification

For detecting different grapevine viruses in the rootstock plants collected, RT-PCR reaction from the previously synthetized cDNA was carried out using "Phire Green Hot start II DNA polymerase". The procedure was handled similarly to the control actin test protocol where the generated cDNA was incorporated to the PCR reagents mixture described in Table 4, instead of the actin primer pair, virus-specific primers either as a single pair (forward and reverse) or combination of two pairs of virus specific primers were added to the PCR master mix. 9.5 µl of the master mix was distributed to the 34 PCR tubes containing each 0.5 µl of the 10x diluted cDNA pool. The amplification was settled up according to the user guide delivered with the Phire Green Hot start II DNA polymerase as described in Table 5. RT-PCR based virus diagnostics were done for the most prevalent grapevine viruses, as listed in the Table 6.

Table 6: List of virus-specific primers used for RT-PCR diagnostics

Virus	Primer name	Primer sequence (5'-3')	At/Tm (C°)	Fragment Length (nt)	Gene	Reference
GFLV	GFLV F GFLV R	ATGCTCCATATCGTGACCCTGT GAAGGTATGCCTGCTTCAGTGG	56	118	RNA 1-polyprotein	Gambino and Gribaudo 2006
ArMV	ArMV F ArMV R	TGACAACATGGTATGAAGCACA TATAGGGCCTTTCATCACGAAT	56	402	RNA 1-polyprotein	Gambino and Gribaudo 2006
GLRaV-1	GLRaV-1 F GLRaV1 R	TCTTTACCAACCCCGAGATGAA GTGTCTGGTGACGTGCTAAACG	56	232	Coat protein	Gambino and Gribaudo 2006
GLRaV - 2	GLRaV-2 F GLRaV-2 R	GGTGATAACCGACGCCTCTA CCTAGCTGACGCAGATTGCT	56	543	Major coat protein	Gambino and Gribaudo 2006
GLRaV 3	GLRaV-3 F GLRaV-3 R	TACGTTAAGGACGGGACACAGG TGCGGCATTAATCTTCATTG	56	336	Coat protein	Gambino and Gribaudo 2006

GCMV	Nepo-B s Nepo-B a	ATGTGYGCHACYACWGGHATGCA TTCTCTDHAAGAAATGCCTAAGA	50	391	RNA 2-polyprotein	Gambino and Gribaudo 2006
GVA	GVA F GVA R	GAGGTAGATATAGTAGGACCTA TCGAACATAACCTGTGGCTC	56	272	Coat protein	Gambino and Gribaudo 2006
GVB	GVB H28 GVBC410	GTGCTAAGAACGTCTTCACAGC ATCAGCAAACACGCTTGAACCG	56	460	Putative RNA Binding protein	Gambino and Gribaudo 2006
GFkV	GFkV F GFkV R	TGACCAGGCTGCTGTCTCTA TGGACAGGGAGTTGTAGGAG	56	179	Coat protein	Gambino and Gribaudo 2006
RSPaV	RSPaV F RSPaV R	GGGTGGGATGTAGTAACTTTTGA GCAAGTGAAATGAAAGCATCACT	56	155	Replicase	Gambino and Gribaudo 2006

F) Forward; **R)** Reverse**; GFLV)** Grapevine Fanleaf; **ArMV)** Arabis mocaic virus; **GLRaV 1-2-3)** Grapevine Leafroll-associated virus1-2-3; **GCMV)** Grapevine Chrome mosaic virus; **GVA)** Grapevine virus-A; **GVB)** Grapevine virus-B; **GFkV)** Grapevine fleck virus; **RSPaV)** Rupestris stem pitting-associated virus.

5.2. Virus Detection approach

To analyse DNA fragments amplified by PCR and detecting the presence of the investigated viruses, agarose gel electrophoresis was the most effective approach to separate the DNA products and enable a clear visualization of the results.

a. Preparation of the agarose gel

1) Measuring 3.6 g of agarose and 300 ml (1x TBE which is a buffer solution containing a mixture of Tris base, boric acid and EDTA)
2) Heating the agarose/buffer mixture in a microwave at 30 s intervals, the content was swirled to mix well. The procedure was repeated until the agarose has completely dissolved.

3) The gel was let to cool down and then, poured into a gel tray with the well comb in place.
4) The newly poured gel was let to sit at room temperature, until it has completely solidified.
5) 0.7 ul Ethidium bromide was added to25 ml of agarose gel (EtBr binds to the DNA and allow the visualization of the DNA under ultraviolet (UV) light).

b. Setting up of gel apparatus and separation of DNA fragments

1) 1ul of loading dye was added to each DNA sample.
2) Once solidified, the agarose gel was placed into the gel box (electrophoresis unit).
3) The gel box was filled with 1xTAE (or TBE) until the gel was covered.
4) A molecular weight ladder was loaded carefully into the first lane of the gel.
5) Samples were loaded into the additional wells of the gel.
6) The gel was run at 80-150 V until the dye line is approximately 75-80% of the way down the gel.

c. Observing separated DNA fragments

1) When electrophoresis was completed, the power supply was turned off and the lid of the gel box removed.
2) Gel was removed from the gel box. Excess buffer was drained off from the surface of the gel.
3) The gel electrophoresis results were screened by a documentation gel system. “Bio-RAD chemidoc MP imaging system”.

3. Results and Discussion

1. Data presentation

1.1. RNA extraction results

The RNA extracted from the 4 distinct plant organs (OdL: old leaf, YgL: young leaf, Flw: flower and Tdr: tendril) of the 34 rootstocks were visualized by gel electrophoresis and RNA concentration measured by NanoDrop Spectrophotometer.

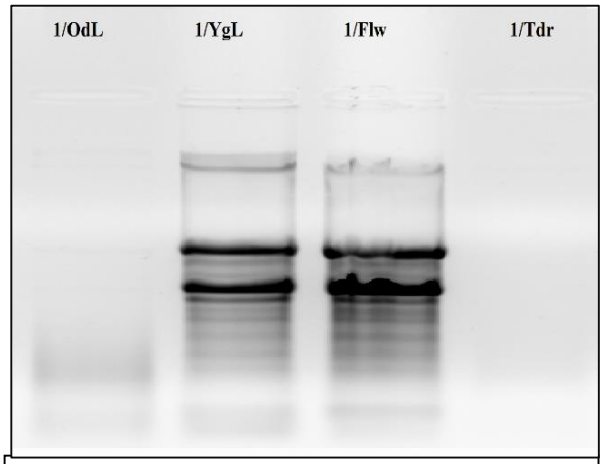

Figure 1: Extracted RNA detection of plant n°1 (Teleki 8B)

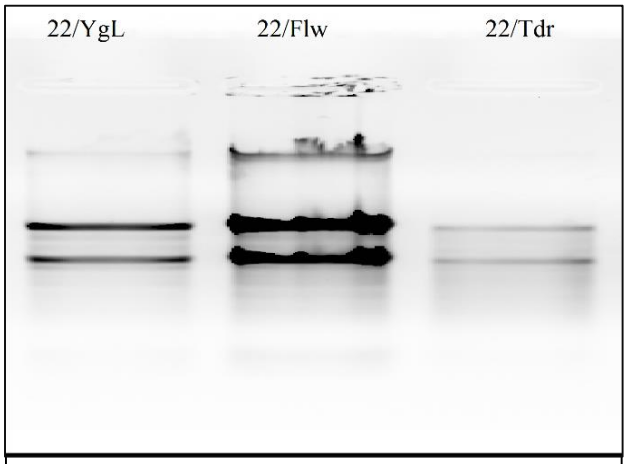

Figure 2: Extracted RNA detection of plant n°22 (Teleki-Kober 5BB Wei.48)

Table 7: Measurement of RNA concentration of Plant n°1 and n°22 measured by NanoDrop

Rootstock Sample		RNA concentration (ng/ml)
Rootstock n°1 (Teleki 8B)	Old leaf (OdL)	279.5
	Young leaf (YgL)	316.8
	Flower (Flw)	198.9
	Tendril (Tdr)	34.3
Rootstock n°22 (Teleki-Kober 5BB Wei.48)	Young leaf (YgL)	192.3
	Flower (Flw)	150.5
	Tendril (Tdr)	164.2

We took example of plant n°1 and n°22 to exhibit the general outlook of the extracted RNA by gel electrophoretic detection (Figure 1 and 2) and NanoDrop Spectrometer concentration measurement (Table 7). Concentration of RNA extracted from young leaf and flower was high, while from old leaf and tendril was very low.(Appendix), As the quality of the RNA is very important for further steps of the analysis (cDNA synthesis), we only used RNA extracts with high concentration and intact bands according to the gel electrophoresis. We could conclude that RNA concentration of different plant organs varied independently of the rootstock species, whereas most of the RNA extracted from young leaf and flower resulted better quality and higher concentration.

1.2. Results of the cDNA quality check

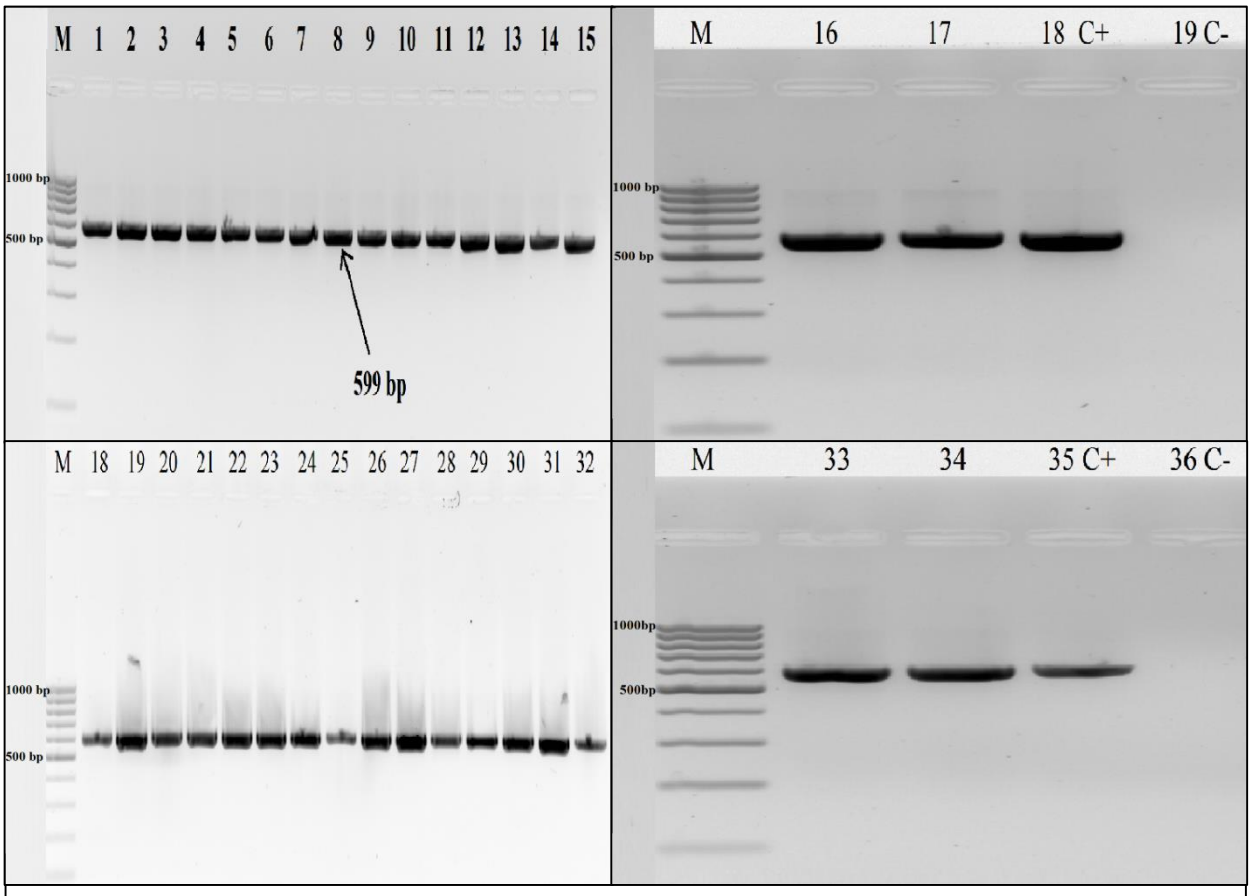

Figure 3: Result of the cDNA quality check – PCR from the synthetized cDNA was done by grapevine actin specific primers. The resulted 599bp product was visualized after separation by gel electrophoresis

In order to test the presence of viruses in our sample by RT-PCR we had to make a proper template for DNS polymerases. The viruses which presence were checked are RNA viruses so

before starting the PCR a cDNA template synthesis was carried out. Good quality RNA, extracted from different organs of the same plant were pooled. cDNA was synthetized using a random hexamer primer – generating cDNA from all host grapevine mRNA and all of the presented RNA viruses. To test the quality of the synthetized cDNA PCR with grapevine actin specific primers was carried out. Figure 3 shows that all of our cDNAs synthetized from the rootstock showed the proper amplified product, while nothing in the negative control (C-), suggesting that cDNA synthesis was successful.

1.3. Virus diagnostics by RT-PCR with virus specific primers

Detection of the virus specific products in the investigated rootstock samples

RT-PCR was carried out using virus specific primers alone or as a duplex. In duplex reaction two specific virus primer pairs were combined in the analysis. This could be done because virus specific primers designed to amplify a part of the combined viruses have the same annealing temperature, but resulted in products of different sizes that made it possible to distinguish which virus is present in the sample. As a positive control we used cDNAs from previous RTs proved to be positive for the investigated virus. In the next chapters we will detail the results according to the PCRs.

1.3.1. Grapevine Leafroll-associated virus-2 (GLRaV- 2) and Grapevine virus-A (GVA) detection results

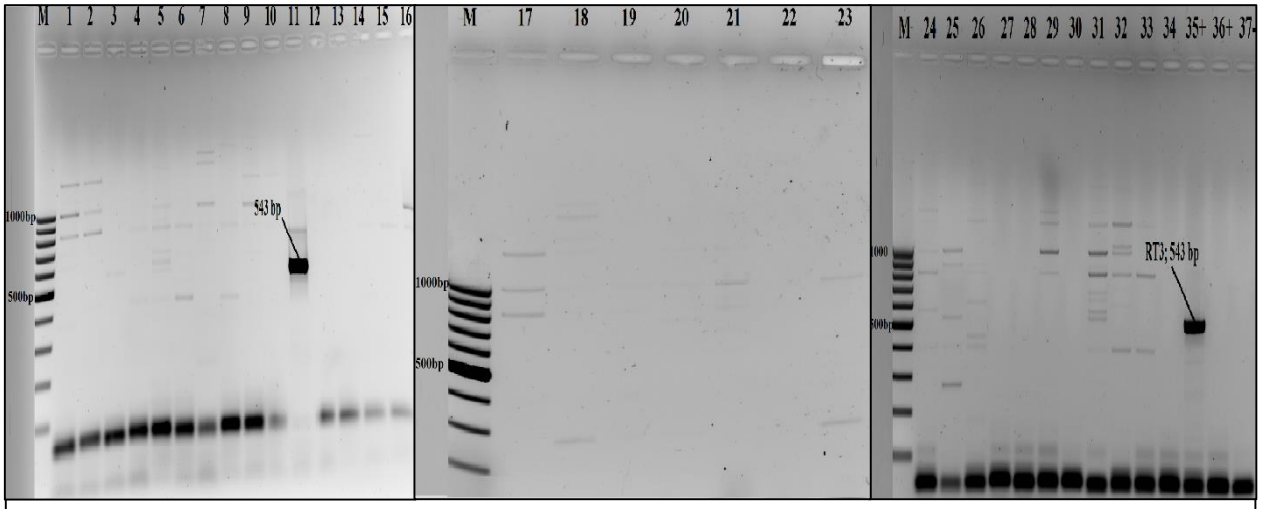

Figure 4: Gel electrophoretic detection of GLRaV- 2 and GVA virus in the rootstock samples investigated

By using positive controls (RT3 for GLRaV- 2 and RT7 for GVA), we could detect the appearance of only one GLRaV-2 infected rootstock n°11 by represented in figure 4, the control RT7 did not appear in the imaging. The lower intensive band is not a virus specific signal, but a typical primer-dimer appearance.

1.3.2. Grapevine Leafroll-associated virus-1 (GLRaV-1) and Grapevine Fanleaf (GFLV) detection results

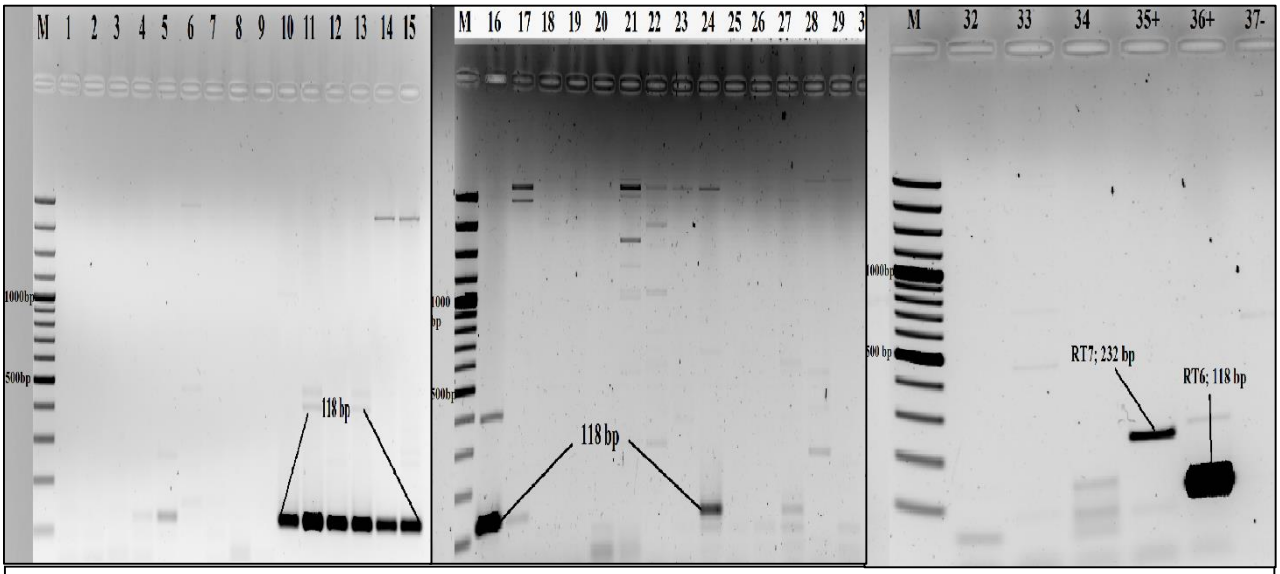

Figure 5: Gel electrophoresis detection of GLRaV- 1 and GFLV in the rootstock samples investigated

As illustrated in the Figure 5, the rootstock samples numbered 10, 11, 12, 13, 14, 15, 16 and 24 indicated a clear presence of the Grapevine Fanleaf (GFLV) which was also detected in positive control used RT6. There were no detected presence of GLRaV- 1 except in the positive control RT7.

1.3.3. Grapevine Leafroll-associated virus-3 (GLaV-3) detection results

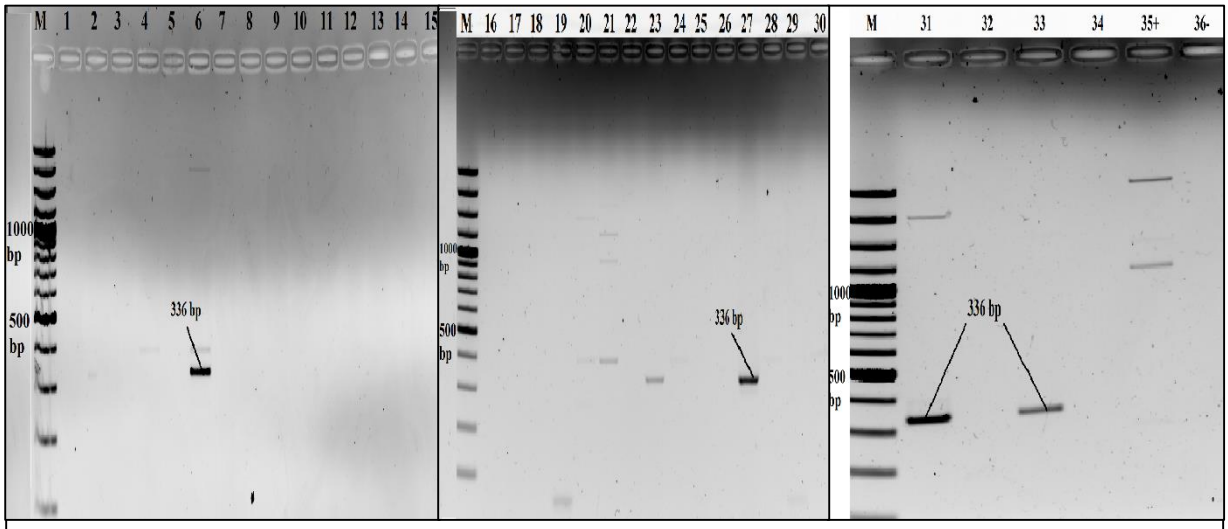

Figure 6: Gel electrophoretic detection of GLRaV- 3 in the rootstock samples investigated

In Figure 6, we can notice the distinct 336 bp fragment in rootstock samples n° (6, 27, 31, and 33) which should be visible also in the positive control RT4 in the 35+ position. The negative control is clean and does not show any contamination.

1.3.4. Grapevine virus-B (GVB) and Rupestris stem pitting-associated virus (RsPaV) detection results

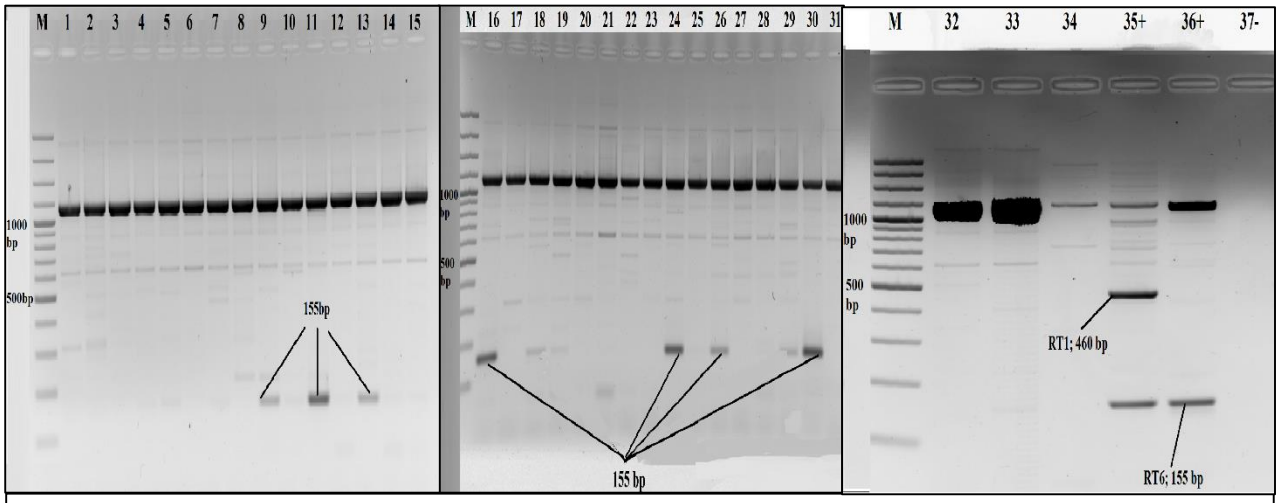

Figure 7: Gel electrophoretic detection of GVB and RsPaV in the rootstock samples investigated

GVB and RsPaV specific products were identified using positive controls RT1 (460 bp GVB) and RT6 (115 bp - RsPaV) respectively. In Figure 7, only plant samples n° 9, 11, 13, 16, 24, 26, 30 showed visible viral fragment of RsPaV, while no infections has been detected as GVB, we can also observe amplification of some high molecular weight product at higher molecular

weight in all the samples except in the negative control which certifies the accuracy of the experiment.

1.3.5. Grapevine fleck virus (GFkV) detection results

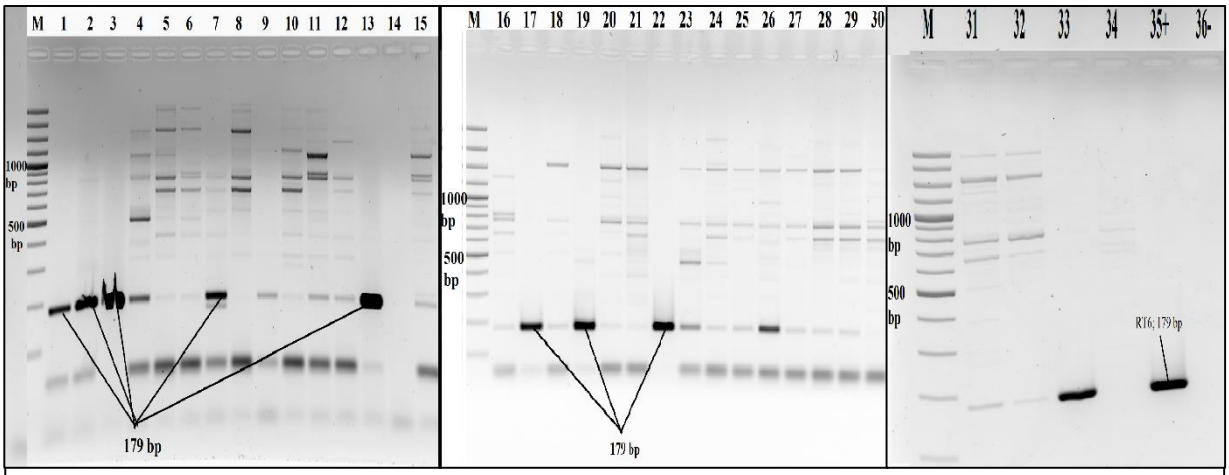

Figure 8: Gel electrophoretic detection of GFkV in the rootstock samples investigated

Considering that the positive control of GFkV RT6 with 179 bp is positioned in 35+, the other 179 bp fragment observed in rootstock n°1, 2, 3, 7, 13, 17, 19, 22, 33 confirms the presence of Grapevine fleck virus in all the mentioned samples where the signal was very strong, but we can also observe that it appears at a low rate in other samples.

1.3.6. Arabis mosaic virus (ArMV) detection results

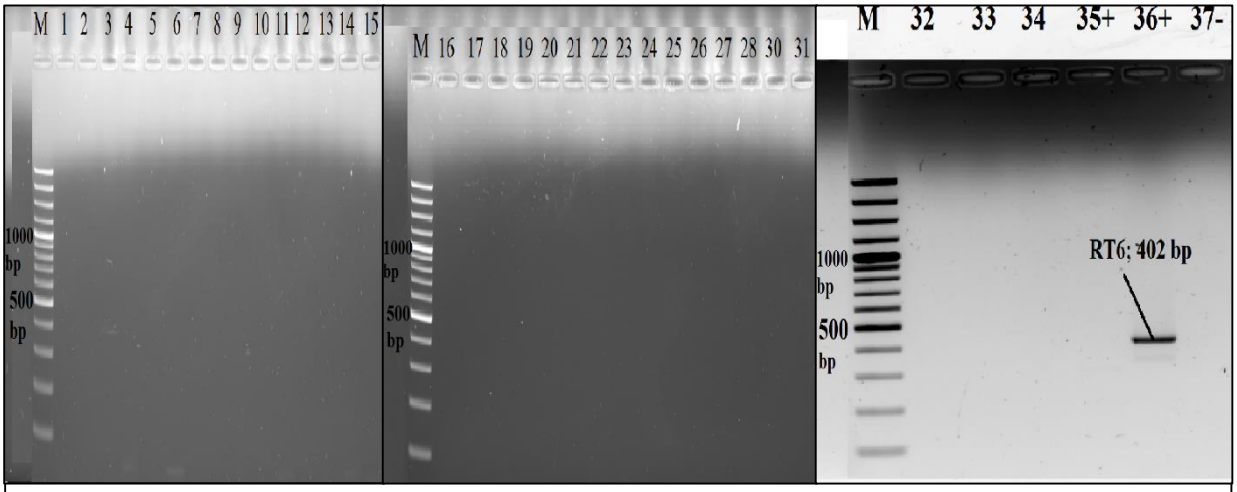

Figure 9: Gel electrophoretic detection of ArMV in the rootstock samples investigated

Essentially, there was no infection of ArMV identified in Figure 9 using the RT6 positive control of 402 bp, and negative control remain clean.

1.3.7. Grapevine Chrome mosaic virus (GCMV) detection results

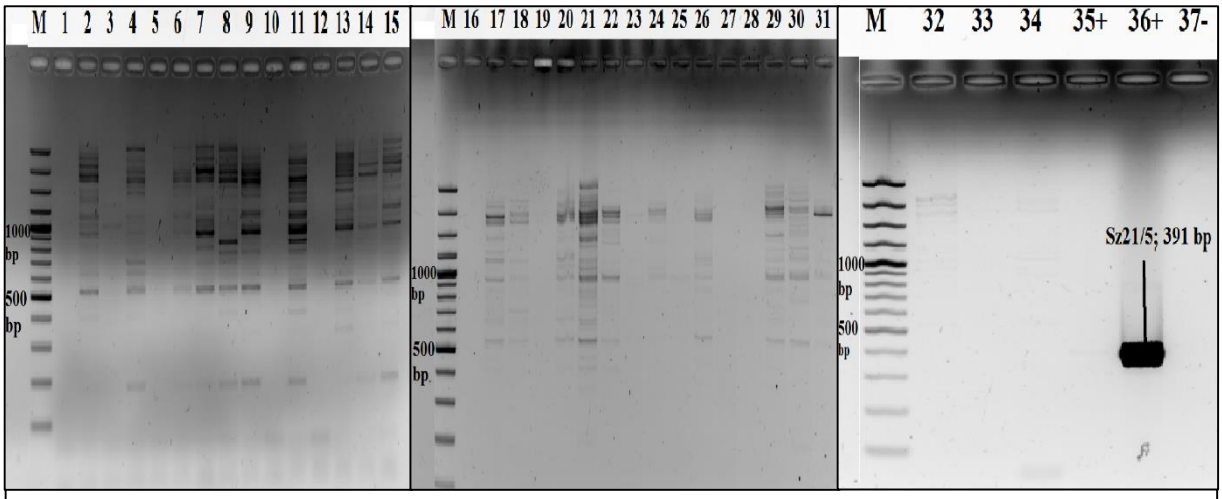

Figure 10: Gel electrophoretic detection of GCMV in the rootstock samples investigated

Investigation of the GCMV infection of the rootstock samples is represented in Figure 10. We can declare that the GCMV specific viral product is absent in all samples, it was only detected in its positive control Sz21/5 (391 bp). A specific product could be generated from the host-grapevine genome by random annealing of the virus specific primers.

2. Interpretation

RNA extracted from the rootstock samples mainly derived from the young leaves and flowers of the plants, due to the low RNA concentration of the old leaves and tendrils which is explained by the low cellular activity of this old plant parts.

cDNA synthesis had been carried out successfully using the actin test which uses 2 primers that anneal prior to the target boarders of the target gene which is 600 bp fragment and amplify it to confirm that the cDNA was synthesized correctly.

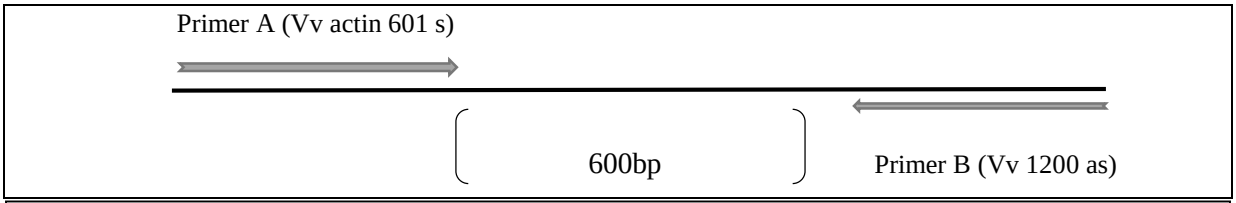

Figure 11: Actin primers flanking target cDNA

To amplify a virus specific fragment from a host RNA sample we have to convert the RNA fragment to the proper template so that DNA polymerases use only DNA as a template.. Contrarily, an RNA polymerase using a short primer, will eventually anneal to the RNA sample and synthetize a DNA strand in a complementary manner to the RNA. In our experiments we used RNA as a template, random hexamer primers for annealing and reverse transcriptase, an enzyme which is able to synthetize cDNA.

Table 8: Results of the virus diagnostics in the rootstock collection investigated

N°	Specific virus / Rootstock variety	GFLV	ArMV	GLRaV- 1	GLRaV- 2	GLRaV- 3	GCMV	GVA	GVB	GFkV	RSPaV
1	Teleki 8B	-	-	-	-	-	-	-	-	X	-
2	Teleki 5C I	-	-	-	-	-	-	-	-	X	-
3	Teleki-Kober 5BB	-	-	-	-	-	-	-	-	X	-
4	Szilagyi 157 Pécs	-	-	-	-	-	-	-	-	-	-
5	Riparia portalis	-	-	-	-	-	-	-	-	-	-
6	Rupestris du Lot	-	-	-	-	X	-	-	-	-	-
7	Rupesris metallica	-	-	-	-	-	-	-	-	X	-
8	Chasselas x Berlandieri 41 B M. et de G	-	-	-	-	-	-	-	-	-	-
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	-	-	-		-	X
12	Rupestris x Berandieri T .10A	X	-	-	-	-	-	-	-	-	-
13	Solonis x Riparia 1616 C	X	-	-	-	-	-	-		X	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	-	-	-	-	-	-	-	-	-	-
19	Teleki 5C Gm. 6	-	-	-	-	-	-	-	-	X	-
20	Teleki 5C Gm. 10	-	-	-	-	-	-	-	-	-	-
21	Teleki-Kober 5BB Gm. 13	-	-	-	-	-	-	-	-	-	-
22	Teleki-Kober 5BB Wei.48	-	-	-	-	-	-	-	-	X	-
23	Teleki 5C wed.	-	-	-	-	-	-	-	-	-	-
24	Teleki-Kober 5BB Fr. 148	X	-	-	-	-	-	-		-	X
25	Teleki-Kober 5 BB	-	-	-	-	-	-	-	-	-	-
26	Teleki 5C II	-	-	-	-	-	-	-		-	X
27	Teleki 5C P	-	-	-	-	X	-	-	-	-	-
28	Teleki-Kober 5 BB P XII.4	-	-	-	-	-	-	-	-	-	-

29	Teleki-Kober 5 BB P XVIII.37	-	-	-	-	-	-	-	-	-	-
30	Teleki-Kober 5BB Cr 2.	-	-	-	-	-	-	-		-	X
31	Borner	-	-	-	-	X	-	-	-	-	-
32	Fercal	-	-	-	-	-	-	-	-	-	-
33	Richter 110	-	-	-	-	X	-	-	-	X	-
34	Richter 140	-	-	-	-	-	-	-	-	-	-

We tested the presence of all described viruses in all of our rootstock samples. The results varied some were infected with more than one virus, while some of them seems to be virus free. The Table 8 summarize the overall results found in the investigated plantation which consist of 34 main rootstock varieties. The diverse sampling was primordial to have a general diagnostic on the virus infection of the different rootstock, where we could assess the sensitivity of the rootstock variety toward one or several of the most prevalent grapevine viruses.

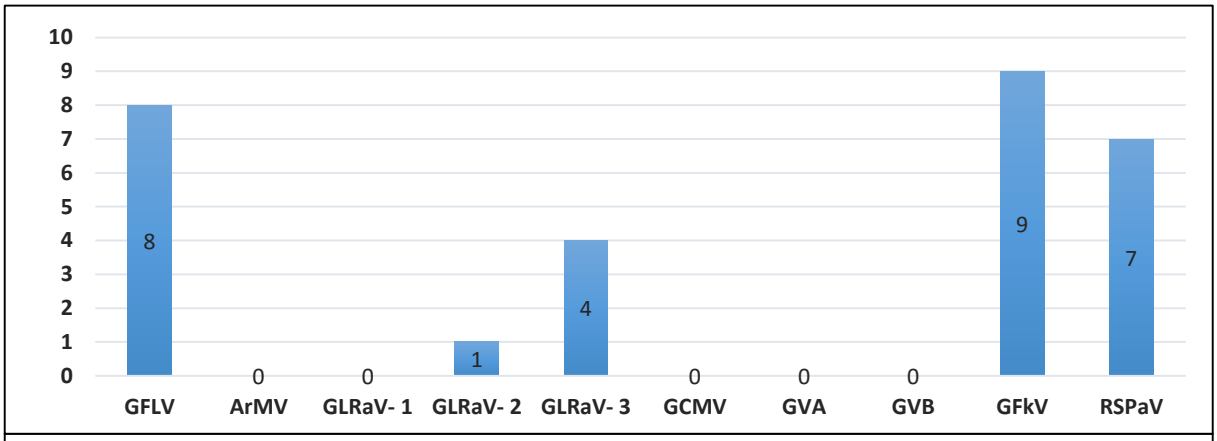

Figure 12: Representation of the rate of virus infection among the rootstock samples studied

As presented in the Figure 12, from the 10 investigated viruses only 5 were detected representing a rate of virus infection of 60%, which reflect the alarming phytosanitary situation of the studied vineyard, knowing that the diagnostic survey was held from a small rootstock sampling of 34 subjects.

Statically, 26.5% of the rootstock population was affected by the Grapevine Fleck disease (GFkV) which is considered to be the highest rate observed, followed by 23.5% individuals which have exposed the presence of Grapevine Fanleaf decline (GFLV), accordingly 20.6% individuals have shown the presence of RSPaV.11.8% of the stock collection displayed Grapevine Leafroll-associated virus-3 (GLRaV-3) and only a 3% of the presence of Grapevine Leafroll-associated virus- 2 (GLRaV- 2) was detected in Mourvédre x Rupestris 1202 C hybrid

rootstock. However, there were no detection observed on the following viruses: Arabis mosaic virus (ArMV), Grapevine chrome Mosaic Virus (GCMV), Grapevine virus-A (GVA) and Grapevine virus-B (GVB)), and we could count 11 from 34 individuals were not affected by any of the specified viruses, representing 32% of the rootstock population studied in contrast to the infected samples rate of 68%. The inability of detecting some particular viruses does not mean that they do not occur in the Hungarian vineyards in general.

The negative results obtained from some of the studied samples may be explained from different point of view, considering several factors that could be responsible for their lack of detection: such as the possibility that the samples which showed no infection for any viruses may contain it but in a low concentration under the sensitivity limit of our diagnostic method. Nonetheless, we can't reject the hypothesis that the 11 non-infected rootstock varieties may have acquire a potential resistance toward the virus investigated or just not have been contaminated which confirm their virus free status.

In that context, we have to mention the rootstock varieties that were not infected by any of the prevalent viruses; Szilagyi 157 Pécs, Riparia portalis, Chasselas x Berlandieri 41 B M. et de G, Teleki-Fuhr S. O.4, Teleki 5C Gm. 10, Teleki-Kober 5BB Gm. 13, Teleki 5C wed., Teleki-Kober 5 BB, Teleki-Kober 5 BB P XII.4, Teleki-Kober 5 BB P XVIII.37, Fercal and Richter 140. Further analysis are needed to deepen our knowledge on the sensitivity and resistance of those particular varieties.

The rootstock varieties that were the most affected by the viruses are Mourvédre x Rupestris 1202 C and Solonis x Riparia 1616 C where both showed GFLV and RSPaV infections and GLRV-1 and GFkV respectively. This multiple infection reflect the high sensitivity of the rootstock varieties toward the cited viruses.

Conclusion

Grapevine cultivation is traditionally established in Hungary for decades. Facing adversity of multiple virus and virus-like infections that influenced negatively the performance of the cultivars at a certain extent, causing the premature death of the stocks and generating great losses in the field. In that context, several studies have been raised to unravel the situation by intensifying the methods of control and detection of viral presence in the vineyard to sustain virus free vineyard and limit their dissemination by infected propagation and grafting materials as it is the most common way of virus spreading.

The study was set out to investigate the viral state of a rootstock collection by using molecular diagnostic method for a specific detection of the most epidemic viruses known in Hungary. We were able to make a conclusive analysis exhibiting the distribution and the rate of viral infections among the diverse rootstock collection inspected, and that contribute in discerning the sensitivity and resistance of the rootstock varieties toward the viruses studied.

Several factors were crucial to the well-establishment of the diagnostic analysis, where the procedures forerunning the virus detection were highly detrimental in providing the adequate information to pursue the examination The quality of the extracted RNA limited the analysis of sampling to young leaf and flower because of their high cellular activity, in addition to the favorable outcome from cDNA synthesis that was conducted with success enabling the amplification of the virus specific primers to anneal to the appropriate host fragment, which eventually permit a clear and accurate diagnostic analysis of the 10 viruses in the 34 vine rootstock varieties.

The detection of the viruses was segregated between the different rootstock samples in irregular ways, Several varieties showed the presence of GFkV, GFLV and RSPaV infections representing a 70% amount of the total rootstock infection which affirm their important dissemination in the investigated vineyard, in contrast to other viruses as ArMV, GCMV, GVA, GVB and GLaRV-1 that were not identified in any sample.

In general, this molecular based survey was very efficient in providing a generic overview on the virus presence and an overall profiling of the stock collection infections toward most prevalent viruses. However some limitation have to be mentioned regarding a potential

contamination when handling the reagents and laboratory utensils which should be taken in consideration due to their misleading effect on the final judgment about the diagnostic accuracy.

Recommendations

After testing and analyzing the main factors responsible for the infections found in the studied stock collection using RT-PCR method, we could attest of the efficiency of the technique and approve it as a reliable but still generic application which may be improved in association with more precise and innovative techniques. Introducing Next generation sequencing to deepen our knowledge on the infection mechanism of the different viruses and the immune system response (interference RNA) of the plant host will open new perspectives in the diagnostic field to detect the presence of viruses and viroids and identify such types which may not have been described yet in Hungary.

References

Adams, I., Glover, R., Monger, W., Mumford, R., Jackeviciene, E., Navalinskiene, M., et al., (2009): Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. Mol. Plant Pathol. 10, 537–545.

Al Rwahnih M., Daubert S., Golino D., Rowhani A., (2009): Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Elsevier publications. Volume 387, Issue 2, Pages 395–401.

Al Rwahnih, M., Daubert, S., Golino, D., Rowhani, A., (2009): Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology 387, 395–401.

Alkowni R., Rowhani A., Daubert S., Golino D., (2004): Partial characterization of a new ampelovirus associated with grapevine leafroll disease. Journal of Plant Pathology 86: 123-133. Virology 155: 1871-1876.

Barba M., Czosnek H. and Hadidi A. (2014): Historical Perspective, Development and Applications of Next-Generation Sequencing in Plant Virology. Viruses 2014. 6. 106-136.

Becker H. (1968): Les porte-greffes Berlandieri x Riparia. Progr. Agric.Vitic. 168, 196-208.

Beuve M., Candresse T., Tannières M., Lemaire O.; (2015): First report of Grapevine pinot gris virus (GPGV) in grapevine in France. Plant Disease 99(2), 293-294.

Bisztray Gy D, Lázár J, Szegedi E, Varga G, Nagy B and Hajdu E. Acclimatized in vitro grapevine plants started from shoot tip cultures 63 material of grapevine and its results in Hungary. International Journal of Horticultural Sciences, 8: 39–43.

Bisztray GyD., Civerolo EL., Dula T., Kölber M., Lázár J., Mugnai L., Szegedi E. & Savka M A (2012): Grapevine pathogens spreading with propagating plant stock: detection and methods for elimination. In: Szabó PV & Shojania J, eds, Grapevines: Varieties, Cultivation and Management, Nova Science Publishers, Inc., 1–86.

Bonavia, M., et al.. (1996): Studies on corky rugose wood of grapevine and on the diagnosis of grapevine virus B. Vitis, 35: 53–58.

Boonham N., Kreuze J., Winter S., van der Vlugt R., Bergervoet J., Tomlinson J., Mumford R. (2014): Methods in virus diagnostics: From ELISA to next generation sequencing. Virus Research 186 (2014) 20–31.

Boscia D., Greif C., Gugerli P., Martelli G.P., Walter B., Gonsalves D. (1995): Nomenclature of leafroll-associated putative closteroviruses. Vitis 34: 171-175.

Capote N., Pastrana AM., Aguado A. and Sánchez-Torres P., (2012): Molecular Tools for Detection of Plant Pathogenic Fungi and Fungicide Resistance, Plant Pathology, Dr. Christian Joseph Cumagun (Ed.). 7: 152-202.

Carstens, E. B. (2010): Ratification vote on taxonomic proposals to the International. Committee on Taxonomy of Viruses. Archives of Virology 155: 133–146.

Chataignier C. (1995) : La Transcaucasie au Néolithique et au Chalcolithique. British Archaeological Series 624: 1–240.

Chien M L., (2008): Grapevine Clones and Rootstocks. College of Agricultural Sciences. Penn State Cooperative Extension.

Choueiri E., Boscia D., Digiaro M., Castellano M. A. and Martelli, G. P. (1996): Some properties of a hitherto undescribed filamentous virus of the grapevine. Vitis 35: 91-93.
Choueiri E., Boscia D., Digiaro M., Castellano M.A., Martelli G.P., (1996): Some properties of a hitherto undescribed filamentous virus of the grapevine. Vitis 35: 91-93.

Cieniewicz E., and Fuchs M., (2015): Grape leafroll disease. Section of Plant Pathology and Plant-Microbe Biology, School of Integrative Plant Science, Cornell University, NYSAES, Geneva, NY.

Clark, M.F., Adams, A.N., (1977): Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34,475–483.

Constable F. and Rodoni B., (2011): Rugose wood-associated viruses. Fact Sheet grape and wine research and Development Corporation.

Crespan M. (2004): Evidence on the evolution of polymorphism of microsatellite markers in varieties of Vitis vinifera L. Theoretical and Applied Genetics 108: 231–237.

Cseh E, Darágó, Á., Takács, A. & Gáborjányi, R. (2011): Survey on the occurrence of grapevine viruses in Hungary. Kertgazdaság, 43: 63–67.

Cseh E, Takács A, Kocsis L, Gáborjányi R (2012): General properties of grapevine viruses occurring in Hungary. Journal of Central European Agriculture, 2012, 13(1), p.44-57

Cseh E., Lázár J., Takács A., Kazinczi G. and Gáborjány, R. (2008): Review of grapevine viruses and virus diseases in Hungary. Növényvédelem 44: 535-544.

Czotter, N., Manduláné F., Lózsa E., R., Ember I., Szûcsné Varga G., Várallyay É., Szegedi, E.,(2015): Primers designed for the detection of grapevine pathogens spreading with propagating material by quantitative real-time PCR. International Journal of Horticultural Science 2015, 21 (1–2): 21–30.

Doazan, J.-P., Rives M. (1967): Sur le determinisme génétique de sexe dans le genre Vitis Ann. Amélior. Plantes. 17 (1967). pp. 105–111.

Donaire L., Wang Y., Gonzalez-Ibeas D., Mayer KF. Aranda MA. & Llave C. (2009): Deep-sequencing of plant viral small RNAs reveals effective and widespread targeting of viral genomes.*Virology.* 392: 203–214.

Dunowska M., Biggs P. J., Zheng T. and Perrott M. R. (2012): Identification of a novel nidovirus associated with a neurological disease of the Australian brushtail possum (Trichosurus vulpecula). Vet Microbiol 156: 418-424.

Engel E.A., Escobar P.F., Rojas L.A., Riveraa P., Fiorec N., Pablo D.T. Valenzuela. (2009): A diagnostic oligonucleotide microarray for simultaneous detection of grapevine viruses. Journal of Virological Methods 163 (2010) 445–451.

EPPO, N.D. Data Sheets on Quarantine Pests Arabis mosaic nepovirus. Prepared by CABI and EPPO for the EU under Contract 90/399003.

Fajardo, Thor Vinícius Martins, Eiras, Marcelo, Nickel, Osmar, Dubiela, Carla Rosa, Souto, Eliezer Rodrigues de. (2012): Detection and partial molecular characterization of Grapevine fleck virus, Grapevine virus D, Grapevine leafroll-associated virus -5 and -6 infecting grapevines in Brazil. Ciência Rural, 42(12), 2127-2130.

Frank T., et al. (2002): Chimerism in grapevines: implications for cultivar identity, ancestry and genetic improvement. Theor. Appl. Genet., 104 (2002), pp. 192–199.

Gambino and Gribaudo, (2006): Simultaneous Detection of Nine Grapevine Viruses by Multiplex Reverse Transcription-Polymerase Chain Reaction with Coamplification of a Plant RNA as Internal Control. The American Phytopathological Society. Vol. 96, No. 11, 2006. 1223- 1229 pp.

Garau, R., et al. (1994): On the relationship between Kober stem grooving and Grapevine virus A. Vitis 33: 161-163.

Giampetruzzi A., et al., (2012): Virus Res. 163:262.

Goldammer T., (2015): Grape Grower's Handbook. A Guide to Viticulture for Wine Production. Apex Publishers. Second Edition. 728 pages

Golino D.A., Sim S.T., Gill R., Rowhani A., (2002): California mealybugs can spread grapevine leafroll disease. california agriculture.vol 56. 6: 169-200.

Gugerli P., Brugger J.J., Bovey R., (1984): L'enroulement de lavigne: mise en évidence de particules virales et développementd'une méthode immunoenzymatique pour le diagnostic rapide. Revue Suisse de Viticulture. Arboriculture et Horticulture 16: 299-304.

Gugerli P., Brugger J.J., Ramel M.E., 1997. Identification immuno-chimique du sixième virus associé à la maladie de l'enroulement de la vigne et amelioration des techniques Journal of Plant Pathology (2012), 94 (1), 7-19

Gugerli P., Ramel M.E., (1993): Grapevine leafroll-associated virus II analysed by monoclonal antibodies. Extended Abstracts 11th Meeting of ICVG. Montreux. Switzerland: 23-24.

Hardie W.J (2000): Grapevine biology and adaptation to viticulture Australian Journal of Grape and Wine Research. 6:74–81

Harrison and Cadman (1959): Nature, Lond. 184: 1624.

Hu J.S, Gonsalves D., Teliz D., (1990): Characterization of closterovirus-like particles associated with grapevine leafroll disease. Journal of Phytopathology 128: 1-4.

Hull R., (1969): Alfalfa mosaic virus. Adv. Virus Res., 15: 365-433.

Jha and Posnette., (1959): Nature, Lond. 184: 962.

Jordan S., (2014): Rugose Wood Complex of Grapevines. February 18, 2014 University of Wisconsin-Madison. Retrieved from http://articles.extension.org/pages/33099/rugose-wood-complex-of-grapevines.

Jordan S.,(2013): Grape Leafroll Disease (November 08, 2013).University of Wisconsin-Madison. Retrieved from http://articles.extension.org/pages/33567/grape-leafroll-disease.

Keller M (2010): The Science of Grapevines Anatomy and Physiology. Academic Press. 1-47 pp.

Kölber, M., Lázár J, Davis R, Dally, E, Tôkés, G, Szendrey, G, Mikulás, J, Krizbai L & Papp E (1997): Occurence of grapevine yellows disease in grapevine growing regions of Hungary. 12th Meeting of ICVG, Lisbon (Portugal), 28 Sept–2 Oct 1997. Extended Abstracts, 73–74

Kreuze, J., Perez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I., et al., (2009): Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses. Virology 388, 1–7.

Lázár J and Bisztray Gy. D (2011): Virus and virus-like diseases of grapevine in Hungary. International Journal of Horticultural Science, 17 (3): 59–63.

Lázár J., Farkas G. né., Farkas, E. and Mikulás J. (1995): A rugose wood complex egyes tagjainak azonosítása Magyarországon, fás indikátorok használatával. (in Hungarian) Növényvédelmi Tudományos Napok, Budapest, Hungary 95.

Lázár, J, Mikulás, J, FarkasG. and Kölber M (2002): Certification program for production of virus-free propagating material of grapevine and its results in Hungary. International Journal of Horticultural Science 8: 39-43.

Lehoczky J. & Reichart G (1968): A szôlô védelme. Mezô- gazdasági Könyvkiadó, Budapest, 41–58.

Lehoczky J. and Farkas, G. (1981): Occurrence of the latent fleck disease of grapevine in Hungary. (in Hungarian) Horticulture 13 (1): 15-25.

Lehoczky J., Martelli G. P. and Sárospataki G. (1969): Leafroll of grapevine in Hungary. Acta Phytopathology Academic Scientenarium Hungarica 4: 117-124.
Lehoczky J., Martelli G.P. & Lazar J. (1992): Seed transmission of grapevine line pattern virus. Phytopathol. Mediterr. 31 : 1 15-1 16.

Lehoczky, J. & Tasnády, Gy. (1971): Fertôzô leromlás és a krómmozaik vírusos kórfolyamatának hatása a szôlôfélék terméshozamára és a bogyók cukortartalmára. Kísérletügyi Közlemények, 64: 49–64.

Lehoczky, J., Kölber, M., Beczner, L. and Pácsa, S. (1984): Distribution of the grapevine chrome mosaic disease in Hungary and detection of its virus (GCMV) in the leaves of outdoor vines by ELISA technique. (in Hungarian) Horticulture 16 (4): 41-51.

Lodhi, M.A., Reisch B.I. (1995): In situ hybridization in Vitis vinifera L.Theor. Appl. Genet., 90 (1995), pp. 11–16.

Martelli and Sàrospataki., (1969): Phytopath. Mediterranea 8: 1, 1969.

Martelli G. P. and Boudon-Padieu E. (2006): Directory of Infectious Diseases of Grapevines and Viroses and Virus-like Diseases of the Grapevine: Bibliographic Report 1998-2004. 1st edition. CIHEAM. Bari, 279 pp.

Martelli G.P., Abou Ghanem N., Sabanadzovic A. Agranovsky A., Al Rwahnih M., Dolja V.V., C.I. Dovas, M. Fuchs, P. Gugerli, J.S. Hu, W. Jelkmann, N.I. Katis, V.I. Maliogka, M.J. Melzer, Menzel W., Minafra A., Rott M.E., Rowhani Sabanadzovic A. and Saldarelli P. (2012): Taxonomic revision of the family closteroviridae with special reference to the grapevine leafroll-associated members of the genus ampelovirus and the putative species unassigned to the family. Journal of Plant Pathology. . Edizioni ETS Pisa, 2012.Vol 94 (1). 7-19 pp.

Martelli, G. P. (1966): Host range and properties of a virus associated with Hungarian grapevines showing macroscopic symptoms of fanleaf and yellow mosaic. Proceedings International Conference on Virus and Vector on Perennial Host with special Reference to Vitis. 402-410.

Martelli, G. P., (1993): Handbook for Detection and Diagnosis- Graft Transmissible Diseases of Grapevines, 1st edition, FAO. Rome, 263 pp.

Martinson T., Fuchs M., Loeb G., Hoch H., (2008): Grapevine Leafroll – an Increasing Problem in the Finger Lakes, the US and the World. Cornell University. Cooperative extension. 1-6p.

Mayo M.A., Robinson D.J., (1996): Nepoviruses: molecular biology and replication. In: Harrison B.D., Murant A.F. (eds.). The plant viruses, polyhedral virions and bipartite RNA, pp. 139-185. Plenum, New York, NY, USA.

McGovern PE., Glusker DL., Exner LJ., Voigt MM. (1996): Neolithic resinated wine. Nature 381: 480–481.

McGovern PE., Rudolph HM., (1996): The analytical and archaeological challenge of detecting ancient wine: two case studies from the ancient Near East. In: McGovern PE, Fleming SJ, Katz SH, eds. The origins and ancient history of wine. New York: Gordon and Breach, 57–67.

Minafra A., Saldarelli P. and Martelli G. P. (1997): Grapevine virus A: nucleotide sequence, genome organization, and relationship in the *Trichovirus* genus. Archives of Virology 142: 417-423.

Monis J., (2009): New test added to Health Check Panel A: Grapevine Syrah virus-1. Eurofins STA Labs. December 2009.

Monis J., (2011): Grapevine Virus Testing Methodologies. Eurofins STA Labs. June 2011.

Monis J., (2012): Detecting Virus on your Vines, a comparison of modern laboratory methods and technologies. Vineyard & winery management. Sept - oct 2012 edition. 74-77.

Mullins M.G, Bouquet A, Williams L.E (1992). Biology of the Grapevine. Cambridge University Press. Cambridge. UK (1992).

Munné-Bosch S (2008): Do perennials really senesce? Trends in Plant Science, 13:216–220

Notomi T., Okayama H., Masuvbuchi H., Yonekawa T., Watanabe K Amino N. & Hase T. (2000): Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research.* 28(12): e63.

Oliver Jonathan E. and Fuchs Marc F., (2011): Fanleaf degeneration/decline disease of grapevines. Department of Plant Pathology and Plant-Microbe Biology, Cornell University, NYSAES, Geneva, NY.

Prabha K., Baranwal V.K and Jain R.K. (2013): Applications of Next Generation High Throughput Sequencing Technologies in Characterization, Discovery and Molecular Interaction of Plant Viruses. Indian J. Virol. (July–September 2013) 24(2):157–165.

Rayapati N., O'Neal S., Walsh D., (2008): Grapevine leafroll disease. Copyright 2008 Washington State University.

Reynolds A., (2015): Grapevine Breeding Programs for the Wine Industry. Elsevier. 103-131 pp.

Robert R., Martin, MacFarlane R., Sabanadzovic S., Quito D., Poudel B., and Ioannis Tzanetakis H., (2013): Viruses and Virus Diseases of Rubus. Plant Disease Feb 2013, Volume 97, Number 2, 168-182.

Robinson J., (2015): The Oxford Companion to Wine. Oxford University Press. Fourth edition. 625-629pp.

Rossetto M., McNally J., Henry RJ. (2002): Evaluating the potential of SSR flanking regions for examining relationships in Vitaceae. Theoretical and Applied Genetics 104: 61–66.

Sabandzovis S. et al. (2001): Complete nucleotide sequence and genome organization of Grapevine fleck virus. Journal of General Virology, v.82, p.2009-2015.

Sefc KM., Steinkellner H., Lefort F., et al. (2003): Evaluation of the genetic contribution of local wild vines to European grapevine cultivars. American Journal of Enology and Viticulture 54: 15–21.

Szegedi E., Ember I., Bisztray Gy., Dula B-né., Hajdu E., Kölber M., Lázár J., Nagy B & Szûcsné Varga G (2012): Integration of elimination and diagnostic methods for the producion of healthy grapevine propagating material (in Hungarian with English summary). Növényvédelem. 48: 469–480.

This P., Jung A., Boccacci P., et al. (2004): Development of a common set of standard varieties and standardized method of scoring microsatellites markers for the analysis of grapevine genetic resources. Theoretical and Applied Genetics 109: 1448–1458.

This P., Lacombe T., Thomas M.R. (2006): Historical origins and genetic diversity of wine grapes.Trends in Genetics, Volume 22, Issue 9, September 2006, Pages 511–519

Thomas M.R., et al.(1993):Repetitive DNA of grapevine: classes present and sequences suitable for cultivar identification Theor. Appl. Genet., 86 (1993). pp. 173–180.

Virus Diseases. (n.d.). Washington state university, viticulture and enology department. Research and extension. Retrieved from http://wine.wsu.edu/research-extension/plant-health/virology/virus-diseases/.

Vunsh, R., Rosner, A., Stein, A., (1990): The use of the polymerase chain reaction (PCR) for the detection of Bean yellow mosaic virus in gladiolus. Ann. Appl. Biol. 117,561–569.

Walsh HA. & Pietersen G. (2013): Rapid detection of Grapevine leafroll-associated virus type 3 using a reverse transcription loopmediated amplification method. *Journal of Virological Methods.*194: 308–316.

Walter B., Cornuet P., (1993): Elisa detection of grapevine fleck virus (GFkV). Agronomie, EDP Sciences, 1993, 13 (7), pp.651-657.

Walter B., Zimmermann D., (1991): Further characterization of of closterovirus-like particles associatd with the grapevine leafroll disease Proceedings 10th Meeting of ICVG. Volos. Greece: 62-66.

Weber E, Golino D and Rowhani A (2002): Laboratory testing for grapevine diseases. Practical Winery and Vineyard. San Rafael.

Zee F., Gonsalves D., Kim K.S., Pool R., Lee R.F. (1987): Cytopathology of leafroll diseases grapevine and the purification and serology of associated closteroviruslike particles. Phytopathology 77: 1427-1434.

Zhang, Y.P., et al. (1998): Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. Phytopathology, 88: 1231–1237.

Zimmermann D., Bass P., Legin R., Walter B., (1990): Characteriztion and serological detection of four closterovirus like particles associated with leafroll disease of grapevines. Journal of Phytopathology 130: 205-218.

Zohary D, (1995): Domestication of the Grapevine *Vitis vinifera* L. in the Near East. P.E. Mc Govern (Ed.). *et al.* The origins and Ancient History of Wine. Gordon and Breach.pp.23–30

Zohary D, Hopf M (2001): Domestication of Plants in the Old World, The Origin and Spread of Cultivated Plants in West Asia, Europe, and the Nile Valley (3rd ed.). Oxford University Press. Oxford (2001).

Zohary D, Spiegel-Roy P (1975): Beginnings of fruit growing in the Old World. Science, 187:319–327

Zohary D. (1996): The mode of domestication of the founder crops of the Southwest Asian agriculture In: Harris DR, ed. The origin and spread of agriculture and pastoralism in Eurasia. London: University College London Press, 142–158.

Zohary D., Hopf M. (2000): Domestication of plants in the Old World, 3rd edn. New York: Oxford University Press, 151–159.

Summary

Thesis title: Virus diagnostic survey of grapevine rootstock varieties from the stock collection of Pecs

Author: Houhou Fakhreddine

Course: Agricultural Biotechnology MSc
Institute/Department: Institute of Genetics and Biotechnology

1. **Primary thesis adviser** (Erzsébet Kiss, Professor, Institute of Genetics and Biotechnology, Szent István University, Faculty of Agricultural and Environmental sciences, Gödöllő)
2. **Independent consultant** (Éva Várallyay, Leader of the diagnostic group, NARIC-ABC, Gödöllő)

Grapevine can be infected by more than 60 viruses but no plant protection against them is possible. They can only be controlled by using virus free propagation material. Grapevine plantation in Hungary are propagated by grafts so phytosanitary status of both the rootstock and the scion variety must be investigated.

Aim of our study was to survey a rootstock collection of the Research Institute of Viticulture and Oenology at Pecs for the presence of the most prevalent viruses in grapevine culture by molecular diagnostic method. .Samples were collected from 34 rootstock varieties from the collection in Pécs. Then, RNA was extracted following CTAB protocol and assembled to create RNA pools mix for each variety from which, cDNA was synthetized and then checked using host specific actin primers in PCRs. Virus detection was carried out applying RT-PCR as the main diagnostic method to analyse the products amplified in a reaction with virus-specific primers. The results were visualized by gel electrophoresis. As a conclusion we can say that we have found viral infection in 29 cases for 5 viruses which represent 60% of the total stock collection. As a summary virus detection survey affirmed our statement on the importance of a prior diagnostic analysis to limit virus proliferation and maintain virus-free rootstock vines.