THESIS

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Investigation of the Causative Agent of a Virus-like Symptom in Grapevine

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ABBREVIATIONS AND ACRONYMS

- **AGVd** Australian grapevine viroid
- **AMV** Alfalfa mosaic virus
- **ArMV** Arabis mosaic virus
- **cDNA** Complementary DNA
- **CEVd** Citrus exocortis viroid
- **CTAB** Cetyltrimethylammonium bromide
- **DNA** Deoxyribonucleic acid
- **ds-RNA** double-stranded RNA
- **EDTA** Ethylenediaminetetraacetic acid
- **ELISA** enzyme-linked immunosorbent assay
- **EtBr** Ethidium bromide
- **GCMV** Grapevine chrome mosaic virus
- **GFkV** Grapevine fleck virus
- **GFLV** Grapevine fanleaf virus
- **GLRaV-1** Grapevine leafroll-associated virus 1
- **GLRaV-2** Grapevine leafroll-associated virus 2
- **GLRaV-3** Grapevine leafroll-associated virus 3
- **GLRaV-4** Grapevine leafroll-associated virus 4
- **GLRaV-7** Grapevine leafroll-associated virus 7
- **GLVd** Grapevine latent viroid
- **GPNV** Grapevine pinot gris virus
- **GRBaV** Grapevine red blotch-associated virus
- **GRSPaV-1** Grapevine Rupestris stem pitting associated virus
- **GSPaV** Grapevine stem pitting-associated virus
- **GSV** Grapevine stunt virus
- **GSyV-1** Grapevine Syrah virus 1
- **GVA** Grapevine virus A
- **GVB** Grapevine virus B
- **GVT** Grapevine virus T
- **GYSVd-1** Grapevine yellow speckle viroid 1
- **GYSVd-2** Grapevine yellow speckle viroid 2
- **HSVd** Hop stunt viroid
- **HTS** high throughput sequencing
- **LR** Leafroll associated virus
- **LTR** long terminal repeat
- **PCR** polymerase chain reaction
- **PHS** Phire Hot Start DNA polymerase
- **PVP** polyvinylpyrrolidone
- **Q5** O5 DNA polymerase
- **RB** Red blotch associated virus
- **RBDV** Raspberry bushy dwarf virus
- **RNA** Ribonucleic acid
- **RpRSV** Raspberry ringspot virus
- **RT** Reverse transcriptase
- **RT-PCR** Reverse transcriptase polymerase chain reaction
- **TBE** Tris base, Boric acid and EDTA
- **TBRV** Tomato black ring virus
- **TMV** Tobacco Mosic virus
- **TRSV** Tobacco ringspot virus

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1. INTRODUCTION

Grapevine is considered as one of the major fruit crops in the world based on hectares cultivated and economic value with a different variety. (OIV, 2018)

Pinot Noir is the grape variety with which many of the world's top winemakers aspire to work: and drinking good Pinot Noir is the ultimate pleasure for many wine enthusiasts. Hungary has some excellent terroirs for producing quality Pinot Noir. There are several leading Hungarian winemakers who have demonstrated their ability to make good Pinot Noir. As far as it was mentioned the first produced in Hungary on the Badacsony State Farm in the 1970's where, it has to be said, it was not very successful. Since then it has been more seriously produced in Eger, Sopron, Pannonhalma, Villány and Pécs. (https.1)

Pinot Noir is a red wine grape of Burgundy. The word Noir comes from the grape skins natural dark color. It is a popular grape that has become even more widespread over the past 40 years. Recent studies show that Pinot Noir is the 10th most widely planted grape in the world! Today there are a total of 117,358 hectares and probably more under vine of Pinot Noir cultivated all over the world. France has the most planted hectares of the grape with 30,351 hectares planted to vine. The United States has the second largest cultivated area with 29,542 hectares under vine.

Pinot Noir is a finicky, less hardy grape requires low yields and is subject to numerous issues in the vineyard that can be brought on by wind, cold or hot weather, fungus or rot, due to its thin skin and susceptibility to disease. The grape does best in cool, dry climates with well drained, stony, or chalk infested soils. (https.2)

It can be infected with several viruses which presence can affect not only its growth, but the quality of important characteristics (berry weight and colour and sugar content, etc.). Grapevine red blotch-associated virus (GRBaV) was described in California, from a vineyard showing red blotch disease using HTS of ds RNAs (Rwahnih et al., 2013) and was proved to be the causative agent of the disease later (Yepes et al., 2018). It is a member of the Geminiviridae family, having a single circular DNA genome and its presence was proved to have inferior effect on berry development (Blanco-Ulate et al., 2017).

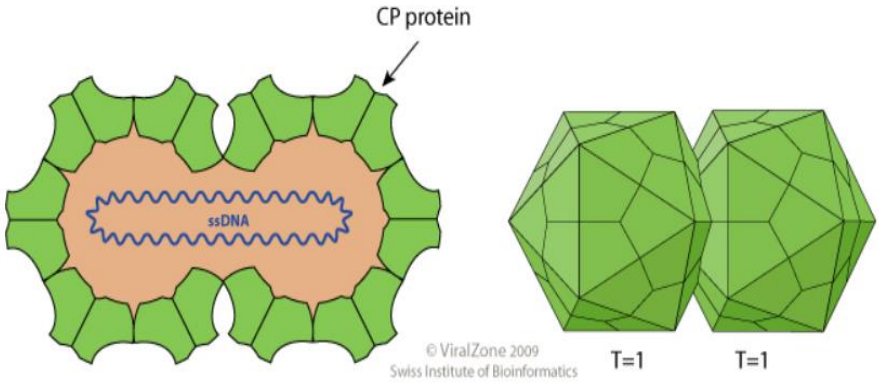

Figure 1: Red-Blotch virus, Non-enveloped, twinned (geminate) incomplete T=1 icosahedral symmetry capsid that contains 22 pentameric capsomers made of 110 capsid proteins (CP). Each geminate particle contains only a single circular ssDNA. (https.3)

The disease symptoms in red varieties include reddening of regions within leaf blades, along with red veins and petioles and delayed fruit maturity. In white varieties, leaves may develop yellow or chlorotic that is similar to leafroll-diseased vines. Asymptomatic vines can remain productive, but they also harbour viruses and act as potential reservoirs for virus spread to susceptible vines. It is graft transmittable, it could originate from a wooded riparian area by a supposed new vector (Cieniewicz et al., 2017). Grapevine virologists highlight that symptoms can be very similar to leafroll disease, with an exception that in GRBaV infected plants margin of the leaf stay flat and instead of green, pink veins appear.

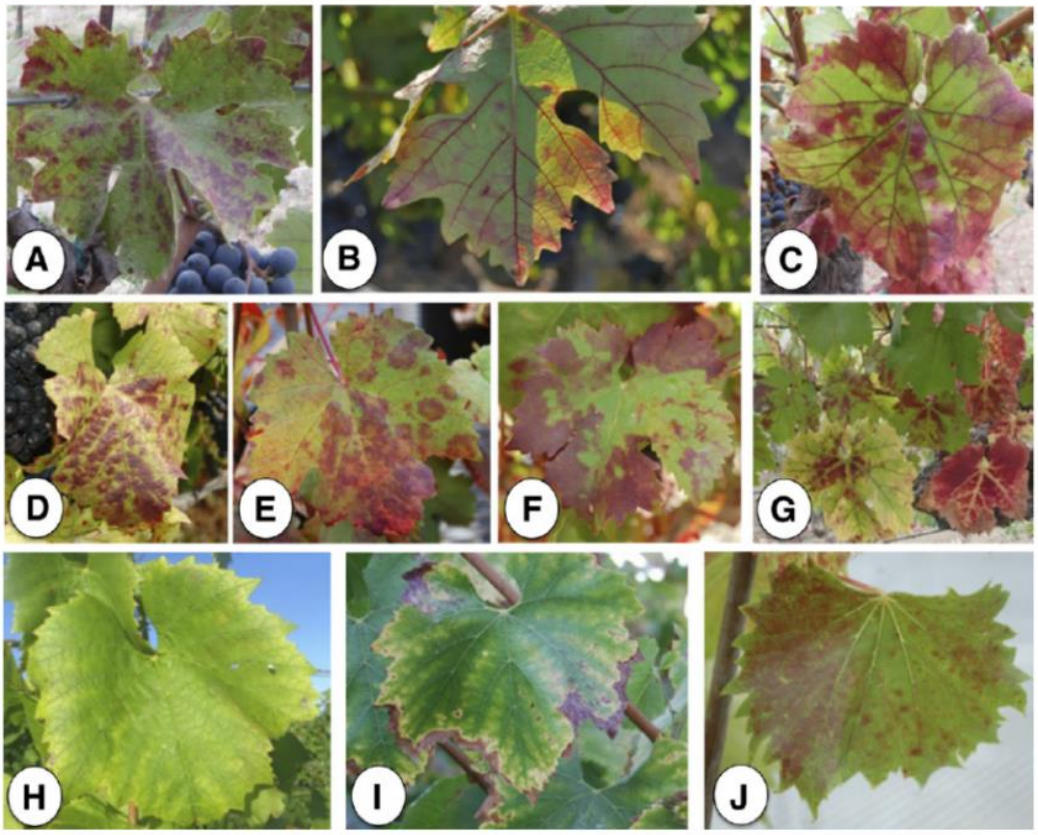

***Figure 2**: Symptoms of Grapevine red blotch-associated virus on Vitis vinifera A, Cabernet Sauvignon (adaxial surface of a leaf); B, Cabernet Sauvignon (abaxial surface of a leaf); C, Cabernet franc; D, Pinot noir; E, Syrah; F, Carignane; G, Merlot; H, Chardonnay with chlorotic areas; I, Chardonnay with necrotic leaf margin; and J, interspecific hybrid Chambourcin. (Sudarshana et al., 2015).*

As small RNA HTS can detect the presence of DNA viruses (Pooggin, 2018), we used this method to reveal the causative agent of the observed, virus-like symptoms.

The Aim of our experiments in this study was to investigate the possible presence of GRBaV in a Pinot noir vineyard showing typical GRBaV specific symptoms.

2. LITERATURE REVIEW

2.1.Grapevine

Grapevine (Vitis spp.) is one of the most important fruit crops grown worldwide. The estimated production in 2014 that there is more than 7.6 million hectares and 74 million metric tons worldwide (MT; OIV 2016). Grapevine based winemaking has been established in old-civilizations of which been found from ca. 6000 BCE.

Grapevine is a climbing plant supported by tendrils that twining onto surrounding structure. Grapevine is also a flowering plant, the flower contains both male and female structure in an inflorescences shape (Clusters), there is around 79 species of family Vitaceae, most of the varieties are wind pollinated. The fruit is non-climacteric type in a berry form, based on variety the berry can be found in green, red, dark blue colour and different size. Colour variation in grape berry is a consequence of an insertion of retrotransposon causing loss of function in the regulation of anthocyanin biosynthesis genes. The wild type grape genome, with anthocyanin synthesis gene, have a dark purple colour. After the mutation, insertion of retrotransposon, the anthocyanin synthesis is blocked resulting in white berry colour. After another mutation, the retrotransposon was removed, anthocyanin synthesis gene function was reversed and became active again. However, a solo LTR of the retrotransposon is remained in the genome, thus influence the function of the main gene which shows light purple/red coloured berry. The berry could be eaten as a fresh fruit or used in food industry: wine, jam, juice, vinegar, raisins, etc., usually the main use is for wine production followed by fresh fruits. (Thompson et al., 2016).

2.2.Pinot noir

Pinot noir is a red to dark blue grape variety of the species *Vitis vinifera*. It is derived from French name for the tightly clustered (small and conico-cylindrical) like pinecone shape bunches of fruit that is associated with Burgundy region of France. The grape is significantly consumed for red wine production, it is among the most popular worldwide wines.

Pinot noir genome was sequenced and announced in 2007 that gave it order for the first fruit crop to be sequenced and the fourth flowering plant. (Minio et al., 2017).

Pinot noir grape skin is thin which makes it easily susceptible to diseases giving it a reputation for being difficult to grow. Although it being popular for its lighter style around 12% alcohol by volume (less alcoholic wines). (https.4)

2.3. Viruses and Viroid of Grapevine

2.3.1. Viruses

The relationship between grapes and virus diseases is similar to that between humans and health problems such as the flu. Observable effects of viral diseases in grapevine range from reduced growth and performance to diminished quality of important characteristics (berry weight and color and sugar content, etc.)

Grapevine virus diseases can destroy crops and cause high costs loss to wine grape producers due to the detrimental effect on the health of the vines, growth, production, quantity and quality of the berries and hence causing agronomic problems.

Virus infections can be difficult to diagnose, and it can be uncertain what damage they cause. Some viruses cause decrease in the vines, although some have quite less economic consequences. Several seriously damaged grapevines are well known, while others are unwell identified or do little or no harm damage.

Viruses are special among plant pathogens because of how they invade their hosts, spread across a plant and how they are transmitted to healthy plants from a diseased one.

Symptoms rely not only on the virus, but the season, the cultivar and other factors that affect the health and efficiency of the vine. Specific symptoms of infections with viruses can be confusing because they can resemble nutritional disorders, herbicide damage, or other non-viral diseases. In several instances, infection may be latent which means the plant is infected but does not have any visible signs.

Until now there are more than 80 grapevine infecting viruses being identified. Around half of these viruses (31 viruses) related to the four main disease complexes known as (1) contagious degeneration (2) leafroll (five viruses), (3) rugose wood (six viruses), and (4) fleck (four viruses). Many of the viruses have RNA genomes with a single strand, some of these viruses have a double-stranded RNA genome, and recently, viruses with a DNA genome have been identified. (Meng et al., 2017)

2.3.2. Viroid’s

Viroid’s are subviral pathogens which have an autonomous reproduction in their host. They consist of 246–375 nucleotides of un-capsulated circular RNA, which size significantly smaller than the smallest viral genome. Viroid’s are categorized into families, genera and species. There

are two recognized families: Pospiviroidae and Avsunviroidae. Their distinguishing features are the existence of a central conserved region in the secondary structure and nuclear replication in the Pospiviroidae) or a branched, ribozyme like secondary structure which lacks a central conserved region and plastidial replication in the Avsunviroidae. Five grapevine-infecting viroids were identified until recently, all of which belong to the Pospiviroidae family: Grapevine yellow speckle viroid 1 (GYSVd-1), Grapevine yellow speckle viroid 2 (GYSVd-2), Australian grapevine viroid (AGVd), Hop stunt viroid (HSVd), and Citrus exocortis viroid (CEVd) (Little and Rezaian 2003). The recent addition to the grapevine viroid list is Grapevine latent viroid (GLVd) (Zhang et al. 2014) and a viroid-like RNA that shares structural features with members of the Avsunviroidae family: grapevine hammerhead viroid, whose biological function in grapevines is yet to be defined (Wu et al. 2012).

It is important to remember that the situation with viruses, viroids and the diseases they cause in grapevine is always complicated due, in part, to the vast number of viruses and their wide number of genetic variants found in mixed infections.

2.4. Red Blotch Virus

Grapevine red blotch associated virus (GRBaV) is a newly discovered grapevine virus and a putative member of a new genus within the Geminiviridae family. This virus is related to red blotch disease which was first recorded in 2008 in California. Symptoms of an infection quite similar to those of leafroll disease, in red-berried grapevine cultivars, symptoms of foliar compose of red blotches early in the season that may spread and coalesce later in the season through most of the leaf surface. In white-berry grapevine cultivars, the symptoms of foliar are less obvious and generally involve frequent chlorotic areas that may become necrotic late in the season.

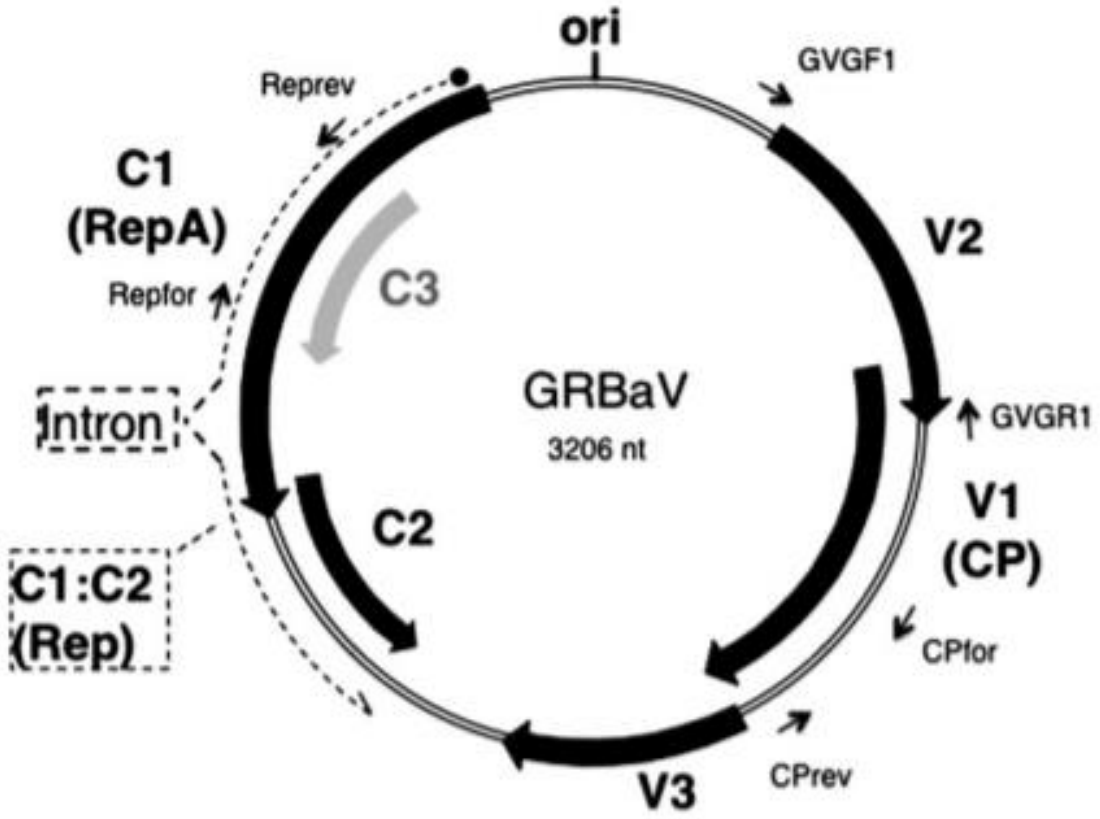

Figure 3: *Genome organization of Grapevine red blotch-associated virus. A circular single-stranded DNA genome of 3,206 nucleotides is shown with predicted open reading frames indicated by thick arrows. The encoded proteins are designated V1, the coat protein (CP); V2, of unknown function; V3, of unknown function; C1, a replication-associated protein (RepA); C2, the C-terminus of the replication protein (Rep), predicted to be expressed from a spliced transcript (shown as a dotted line) and designated C1:C2; and C3, shaded in gray, because it is internal to C1 and in the same reading frame. The ori is a nonanucleotide sequence that functions as an origin of replication. Relative positions of primers used in the diagnostic polymerase chain reaction assays are labeled and indicated with small arrows.(Sudarshana et al., 2015)*

The GRBaV genome consists of a single circular ssDNA molecule (Al Rwahnih et al. 2013; Krenz et al. 2012a, 2014; Poojari et al. 2013; Seguin et al. 2014). The resulting genomic organization, coding potential, and origin replication origin are identical to those of members of the Geminiviridae family, but the 3,206 nucleotides long genome is 4 percent larger than the largest recorded geminiviral genome and only shares 50 percent of the overall sequence identity.

2.5. Transmission of grapevine viral pathogens

Grapevine is frequent target for different viruses. The consequences of persistent infections are decreased yield and efficiency, shortened development periods, weakened rooting of propagation materials reduced disease resistance to abiotic and biotic stressors and, at least, early grape dieback. Several studies have shown that vertical virus transmission is a significant phenomenon affecting a diverse range of viruses, especially as a result of breeding programs. Nevertheless, the main response factors are grafting, vectors, seed transmission and more importantly vegetative propagation.

2.5.1. Grafting

When a scion is grafted onto a rootstock and one of the components of the graft is contaminated with virus, virus can move from the diseased portion to the safe one through the graft site. Grafting as a standard method of producing propagative material is responsible for much inadvertent spread of the virus. Grafting, as a form of virus inoculation, may be implemented if mechanical inoculation (phloem-limited or highly dysfunctional viruses) is difficult and no biological vector is recognized or accessible. Tissues must be compatible for proper union, which ensures the components of the graft should be from the same or similar species. Nevertheless, it is not always important to merge the grafted tissues, since even close touch between two cut surfaces and the resulting exchanging of secretions will contribute to transmission of viruses. (Dijkstra et al., 1998)

Graft inoculation was used in programs which target to:

- Diagnosis of new virus diseases
- Virus identification in crop plants or in propagative material stocks (indexing)
- Generation or development of virus-free material for plants
- Characterisation and examination for virus resistance
- Research on the distribution of viruses within the plant

2.5.2. Vectors

Plants are sessile and their cells are shielded by a cell wall, why plant viruses can hardly spread by touch in nature and use almost always vectors for their spread. Much of these vectors are insects: aphids, leafhoppers, thrips, beetles but they may be mites, nematodes, or fungi. Such

bug mouthpieces are needle-like organs that are designed to pierce plant tissues and draw the contents of plant cells or the water.

During salivation, viruses picked up by hemipteran vectors are then immediately inserted into the plant tissue, enabling them to defeat the first plant defence: the outer cell layer.

These insects may often fly long distances (from a few meters to hundreds of kilometres in air currents) and also feed on various plant species, providing several transport options for the virus they bear.

Transmission of Phyto-viruses may be classified into three major groups, based on how they communicate with the vectors concerned:

Non-circulative transmission in which the virus does not enter the vector's inner body and is preserved and expelled from the anterior alimentary tract, Non-propagative circulatory transmission in which the virus circulates inside the vector, transferring from the gut to the salivary glands without replication, Circulative propagative transmission where the virus circulates in a similar manner but often replicates in the vector.

2.5.3. Seeds

The importance of global seed exchange to modern agriculture is strongly emphasised, in breeder's wide range of crop species, which lead to increased production, long shelf life, pathogen resistance, and tolerance to severe circumstances around the world. Seed transmission of plant viruses has a tremendous epidemiological significance which causes outbreaks of disease worldwide. New crop varieties are brought into new growing areas in modern agriculture and are cultivated in the vicinity of indigenous crops, vulnerable to imported remote endemic diseases.

Seed transmission are two types: horizontal transmission which the plant virus is transmitted as a result of an external source i.e. mechanical means, plant injury, or vertical transmission by which the virus is inherited from a parent, i.e. seed embryo infection by virus infected plant. (Gasparro et al., 2016)

Seed-borne plant viruses pose a threat to world agriculture, however most of the viruses are excluded from the meristem, why they don’t get into the generative part of the plant, and seed transmission does not occur in these cases. Among them, species belonging to the genus Tobamovirus are considered a major danger to a number of cultivars, primarily those belonging

to the families Solanaceae and Cucurbitaceae. For a long time, such viruses were a danger to agriculture. The Tobamovirus viral particles are highly stable, and infectivity in seeds is maintained for up to many years.

Virus seed transmission occurs primarily via infected embryos, through paternal or maternal pathways. Transmission of the virus happens mainly through mechanical means by seedlings transplantation and root cuts that are then vulnerable to contamination by the infected seed cover.

RNA viruses have a significant degree of mutation that leads to rapid processes of evolution that can guarantee adaptation to new host plants, or to stressful and fluctuating conditions. However, seeds have regulatory mechanisms that may inhibit the spread of viruses, and virus spread is blocked by dominant resistance genes.

2.5.4. Vegetative propagation

Vegetative propagation is an asexual, vegetative process present in plants. It exists naturally in certain plants and can be made artificially. To produce genetically identical plants and maintain feature of a certain cultivar cuttings may be taken from so-called "mother plants" and rooted in or can be grafted onto a rootstock. Vegetative propagation raises the amount of material that can be economically sold, which thus improves the possibility of viral plant infection. (Kraus et al. 2008). Vegetative propagated crops are especially vulnerable to virus infections, and this is valid also for plants propagated utilizing plant tissue culture techniques. However special techniques of in vitro cultures can be used during virus release of virus infected mother plants.

Establishment of vineyards are based on vegetative propagation, why viruses can be transmitted immediately during this process if the propagation material is not tested negative for the presence of devastating viruses. The consequences of persistent infections are decreased yield and efficiency, slowed in productive time, poor rooting of propagation materials, decreased disease tolerance to abiotic and biotic stressors, and at least early grape dieback.

2.6. Grapevine-infecting viruses (Table – Virus Families)

Table 1: *A brief description about the viruses.* *(Basso et al., 2016)*

Family	Genus	Species
Viruses with isometric particles +ssRNA genome		
Secoviridae	*Nepovirus*	*Grapevine fanleaf virus* (GFLV). *Raspberry ringspot virus* (RpRSV)
Tymoviridae	*Marafivirus*	*Grapevine Syrah virus 1* (GSyV-1)
	Maculavirus	*Grapevine fleck virus* (GFkV)
Viruses unassigned to Families	*Idaeovirus*	*Raspberry bushy dwarf virus* (RBDV)
Viruses with filamentous particles +ssRNA genome		
Closteroviridae	*Closterovirus*	*Grapevine leafroll-associated virus 2* (GLRaV-2)
	Ampelovirus	*Grapevine leafroll-associated virus 1* (GLRaV-1); *Grapevine leafroll-associated virus 3* (GLRaV-3); *Grapevine leafroll-associated virus 4* (GLRaV-4)
	Velarivirus	*Grapevine leafroll-associated virus 7* (GLRaV-7)
Betaflexiviridae	*Foveavirus*	*Grapevine stem pitting-associated virus* (GSPaV)
	Trichovirus	*Grapevine pinot gris virus (*GPNV)
	Vitivirus	*Grapevine virus A* (GVA); *Grapevine virus B* (GVB)

Viruses with a DNA genome		
Geminiviridae	Undetermined	*Grapevine red blotch-associated virus* (GRBaV)
Ill-defined, taxonomically unassigned viruses		*Grapevine stunt virus* (GSV)

2.7. Virus Diagnostic

Biotests as Grapevine virus disease identification technique depends on detailed responses of certain indicator plants to particular viruses. One of the first uses of pathogen identification with biological assays was in viticulture. While biotest can show only that there can be any causative agent of a symptom ELISA, PCR, or RT-PCR can validate the presence of well-characterised species. The more modern high-throughput sequencing (HTS) methods, as they based on sequencing all of the genetic material in a sample, are able to identify the presence of any viruses including both well-characterized and new ones. In general, such molecular-based methodologies are more accurate, more flexible, and faster than bioassays. However, the current modern laboratory analyses also cannot fully substitute the traditional biological test. The bioassay is also kept using to display the existence of suspected transmissible corruption agents.

2.7.1. ELISA

The enzyme-linked immunosorbent assay (ELISA) is a method that identifies substances such as peptides, enzymes, antibodies and hormones utilizing antibody binding specificity.

Its usage is now very widespread in the identification of plant viruses, with antibodies specific to the target virus coat protein. Engvall and Perlmann (1971) first described it.

The most widely used format is enzyme-linked immunosorbent assay (ELISA) which provides a reliable, sensitive, and quick method for screening large numbers of field samples. A variety of organizations offer ELISA kits of high quality against several of the major viral pathogens that attack grapevines. ELISA offers accurate treatment when samples are obtained in the specified vine tissue at the appropriate period.

Standard grapevine virus screening using ELISA tests and internationally accepted indicator species began in 1972. ELISA has been regularly used since 1985 to identify 7 viruses: *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine leafroll-associated virus* 1, -3

(GLRaV-1, -3) and *Alfalfa mosaic virus* (AMV). ELISA results should be followed by molecular testing in sensitive cases, and vice versa, since certain virus strains cannot be identified by one or another form of test.

While this technique is responsive and accurate, it has some drawbacks, including its inability to detect low titer grapevine viruses, the absence of antibodies for some significant viruses, and the difficulty of manufacturing these reagents.(Osman et al., 2008)

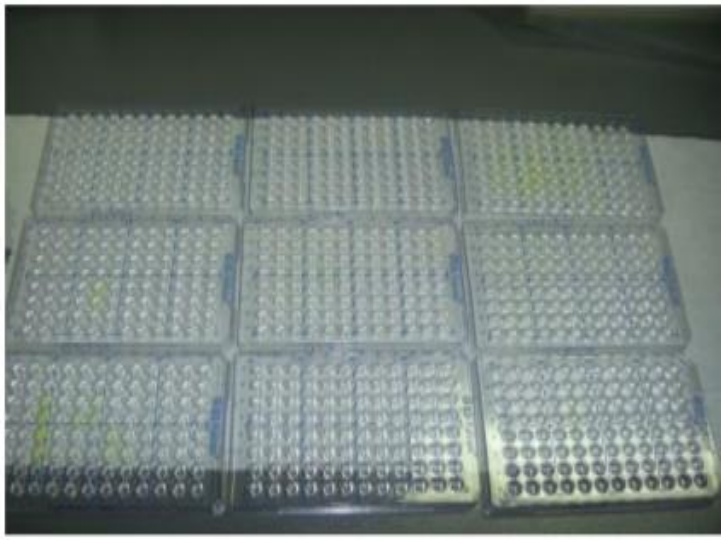

Figure 4: *ELISA Plates to detect Grapevine Viruses. (Dida et al., 2017)*

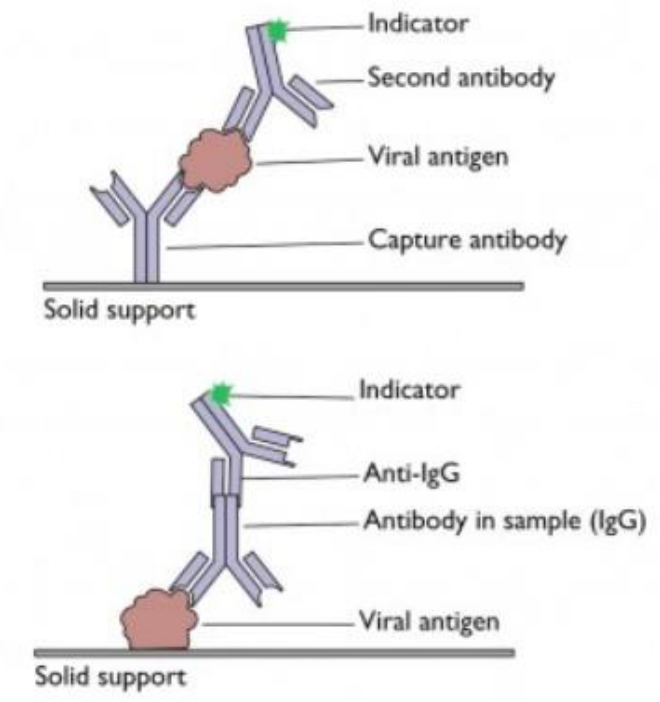

Figure 5: *ELISA Principle. (http.5)*

2.7.2. RT – PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) is a fast, highly responsive, and precise tool for detecting viruses and has been shown to be an effective technique for detecting grapevine viruses (Rowhani et al., 2000).

The advantages of this technique over conventional RNA measurement methods include its sensitivity, wide dynamic range, and the ability for high and precise quantification throughout..(Huggett et al., 2005)

The method includes the isolation of viral RNA from the biological sample, reverse transcriptase conversion to DNA, PCR amplification and amplified DNA identification.

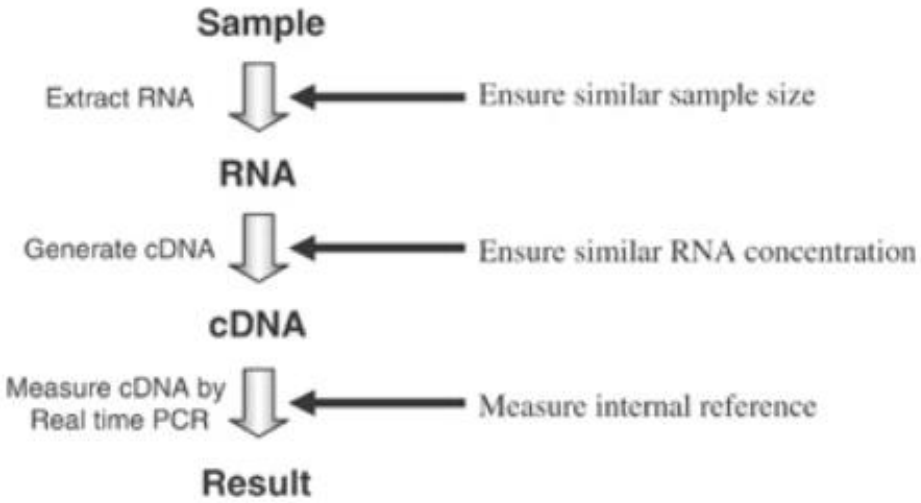

Figure 6: *RT-PCR strategy, Black arrows show points that should be taken into consideration for a successful strategy of normalization. (Huggett et al., 2005)*

Preparation of templates is critical in the detection methods based on the reverse transcription polymerase chain reaction (RT-PCR). Purified complete RNA or double-stranded RNA (ds-RNA) was used as the basis for cDNA synthesis in most RT-PCR-based methods.

2.7.3. Biotest

The method of detecting plant viruses is to inoculate the herbaceous indicator plants with plant sap (Rydén 1977). Biotest may not be a very sensitive procedure in the form of sap inoculation for particular indicator plants and does not detect all the different strains equally well. Indicator plants are plants that, when inoculated with plant viruses, demonstrate clear local and systemic symptoms. Plant extracts to be examined can contain compounds that may inactivate the virus or have an inhibitory effect on the replication of the virus as the pH value drops, as when the cells are smashed. To avoid this, it is necessary to preserve the extract 's high pH and to dilute

it to reduce the effects of inhibition. A phosphate buffer with a pH of 7 to 8 is the most popular substance to use during extraction. Indicator plants are mostly placed 1-2 days prior to inoculation in a dark chamber to make them more vulnerable to infection. An abrasive is applied to an extract to allow the inoculation of virus particles. The abrasive can inflict tiny epidermis wounds that will act as the virus particles' entrance holes. By wiping it softly onto the leaves of the indicator plants, the inoculum is transferred. The leaves are flushed with tap water after inoculation to remove excess sap and abrasive sap (Németh, 1986). (Sjöberg, 2006). However, this method sometimes fails in case of grapevine infecting viruses.

2.7.4. High Throughput Sequencing

High throughput sequencing (HTS) is a sequencing method where millions to trillions of nucleotide sequences can be determined during a single reaction.

Using this technique for virus diagnostics is very straightforward as determines genomic sequences of the viruses and other pathogens in the investigated sample. HTS research is found to be preferable to the regular bioassay for the identification of agronomically relevant viruses, including low-titer virus infections and also because its fast speed. This automated method provides the entirety of both the genetic DNA and RNA sequences in a sample as results, but these sequences has to be further processed by computer-based bioinformatics methods. Pathogens are recognized with the help of the data set with the help of currently available databases with all recognized genomic pathogen sequences. HTS allows quick sequencing of nucleotides in DNA or RNA samples, however as sequencing chemistry only allows to sequence DNA, RNA has to be transformed into DNA before that with reverse transcription. Supporting a vast variety of applications, including gene expression profiling, chromosome counting, epigenetic change detection and molecular analysis, HTS drives exploration and facilitates the potential of precision medicine. Technologies and the vast amount of sequence data thus produced in bioinformatic analysis have dramatically changed this situation. Nonetheless, the detection of any viral agent through HTS of nucleic acids from a host and the identification of viral sequences of known or unknown agents in the sequences produced is now conceptually feasible.

As a first-generation sequencing technology, it could have 96 or 384 sample sequences per instrument with a read length ranging from 600 to over 1,000 nucleotides. This technique went through an enormous transition at the turn of the century, opening the way for the advent of

second and third-generation sequencing technologies, which are commonly referred to as HTS innovations. The key difference between technologies of the second and third generation is that the former involves template amplification before sequencing, while the latter uses individual DNA molecules as a template; Consequently, third-generation sequencing is also referred to as single-molecule sequencing (Rhoads and Au 2015;).

Three common steps are shared by HTS platforms: DNA fragmentation to build a library; the insertion of synthetic DNA adapters to individual fragments; and each fragment sequencing. Noticeably, once RNA is being used as a starting material, a cDNA library is construct firstly by the reverse transcribed process from the fragmented RNA. In addition, RNA selection is performed before the construction of the library. In general, various HTS platforms can be listed based on (i) the method of nucleotide sequence is detected, (ii) the contiguity source of the nucleotide, and (iii) the sequencing chemistry engaged (Levy and Myers 2016). (Villamor et al., 2019)

2.8. Managing viral

Grapevine (Vitis spp.) is a major international world-wide vegetative fruit crop of high socioeconomic significance. It is vulnerable to many graft-transmitted pathogens, causing several diseases and major crop losses, decreasing the quality of fruit, plant vigor and minimizing the lifespan of vines. The spread of these pathogens and the regular sharing of propagative material between countries contribute to the spread of these pathogens, thus facilitating the emergence of complex diseases. The blending and incorporation of multiple viral agents into a single plant is further accelerated by its perennial life cycle. (Basso et al., 2016). Researchers and growers of wine grape are discovering more about the impacts of viruses on vine safety and grape production, contributing to improved guidelines for grapevine virus control. When a plant gets a virus / viroid infected, little can be done to recover its health. Control is carried out using a range of methods, such as cultivating resistant species and plant varieties or acquiring virus-free crops, cuttings or plants as a part of indexing and certification programmes.(Meng et al., 2017)

The primary prophylactic measure for reducing the effect of virus diseases is the good health status of propagative material (cuttings, grafts, buds, rooted cuttings, and grafted plants) (Oliver; Fuchs, 2011). In areas where vectors are present mainly, the production and use of certified virus tested propagative material reduce the inoculum potential (Martelli, 2014).

Setting up vineyards in areas free of vectors decreases the local and long-distance dispersal of viruses. (Laimer et al., 2009; Villate et al., 2008). Measures such as roughing (and cutting any residual roots) of symptomatic grapevines and probably adjacent plants, chemical or biological control or insect- or nematode-vector management, and cross-protection and traditional or transgenic virus-tolerant or nematode-vector-resistant grapevines are potential strategies for the control of viral diseases. (Almeida et al., 2013). Chemical control of nematodes is often not useful, environmentally unsuitable and hazardous to humans, whereas transgenic plants may be a future choice. (Laimer et al., 2009). Thermotherapy in vivo or in vitro (Krizan et al., 2009;), chemotherapy (Luvisi et al., 2011), meristem and shoot tip culture (Maliogka et al., 2009), somatic embryogenesis (Borroto-Fernandez et al., 2009), electrotherapy and cryotherapy (Bayati et al., 2011), are the key sanitation methods to control grapevine viruses. The thermo- or chemotherapy associated with meristem and shoot tip culture has achieved greater efficiency in obtaining virus-free grapevines. (Basso et al., 2016).

2.8.1. Start with Clean Planting Materials

Limiting the passage of contaminated planting material by not planting them in new vineyards or utilizing them as substitutes in established vineyards is the most effective method for avoiding grapevine virus diseases. Because certain virus diseases have asymptomatic, and symptoms in dormant canes are also not evident, check the planting stock supplies with accurate PCR-based and indexing assays to verify that the products are virus-free is essential. Buying "safe" seeding materials from approved nurseries for new vineyards can also help.

2.8.2. Diagnosis

Diagnosis is the first step towards an effective treatment of viral diseases. Their symptoms are often quite furtive, do not occur immediately after transmission, and can be related to other health conditions such as shortages of nutrition, chemical toxicity and severe environmental conditions. Virus symptoms on dormant grapevine canes cannot be noticeable, even though the source vine is contaminated.

Viruses seldom destroy grapevines; however, they can degenerate vines sufficiently over time to contribute to other issues. Virus infection can minimize investment returns due to decreased fruit yield and quality, as well as shorten the viticulture's productive lifetime.

At the time of infection, the intensities of the virus-induced symptoms depend on the species or strain of the virus, scion cultivar, vine age and rising conditions. Symptoms vary from season to season. Some of the symptoms caused by the virus are not turn up before the crop approaches. In conjunction with consulting with a reputable plant diagnostic laboratory, diligent observations made in the vineyard over time are critical for the assured diagnosis of grapevine virus diseases.

Definitive viral recognition can include serological (Enzyme-Linked Immunosorbent Assay or ELISA) or molecular (conventional or reverse transcription polymerase chain reaction [RT-PCR]) methods, accessible by diagnostic laboratories for plant disease. As 2.7. section. The quality and durability of test findings rely on having the right type of tissue samples and submitting them in excellent condition for research.

2.8.3. Remove Infected Vines

Extracting or removing reported contaminated vines may be part of the overall strategy for handling the virus disease. This approach is particularly helpful if the risk of contamination with the vineyard is low and the vineyard is young. Removal of contaminated vines decreases the chance of transmitting virus to other vines inside and beyond of the vineyard block. When determining how to eradicate contaminated vines, potential factor for virus transmission to other uninfected vines and cost-benefit replanting calculations.

2.8.4. Vector Management – Insect and Nematode Control

Control of possible virus vectors including mealybugs, scale insects, nematodes, and hoppers is also part of the comprehensive virus disease control plan. Use vector control chemical agents works better via chemigation (applying chemicals through irrigation water), use approved pesticides for use on grapevines. Because of the possibility of pathogen transmission, retaining lower pest levels for virus vectors is important. Plants are available which are resistant to certain grapevine viruses. For example, Vitis labrusca and V. riparia scion cultivars or root stocks are immune to nepoviruses (such as TRSV), based on their resistance to nematode.

2.8.5. Virus-Free Plants Through In vitro Culture

The technique for producing virus-free plants from an infected stock or plant is in vitro culture.

The shoot and root apices of virus-infected plants have been found to be often devoid of viral particles or to contain very low viral concentrations. In infected leaves, unequal virus distributions have been revealed. It has also been found that not only callus tissues originating from virus-infected plants do show a lower virus title than their source plant, but also contain a high percentage of healthy cells in an infected cell network. Shoot meristems and callus cultures become desirable sources of contaminated stocks to regenerate virus-free plants. These *in vitro* techniques could also be used for the generating of pathogen-free plants from plants systematically contaminated with pathogens other than viruses such as phytoplasma, fungi and bacteria. The significant increase in the regeneration of virus-free plants by *in vitro* culture methods in recent years is evidenced by the massive work published.

2.8.5.1. Virus-Free Plant Regeneration through Meristem Cultures

Meristem cultures include the part of the stem tip meristematic area and the propagation of it on a nutrient medium for plantlet regeneration. The often-used explant is the apical dome (apical meristem), while one or multiple young leaf primordia from the subapical area are also used in most cases. The number of primordial leaves used depends on the species of the plant and the intent of the study. Some workers have a narrower definition and refer only to the culture of meristem as the culture of the apical dome. Other workers can also provide in their explant a portion of the stem outside the meristematic subapical portion. Other words are also used, such as 'meristem tip culture,' tip culture, culture of shoot apices, and shoot apex culture. The value of using a meristem culture as a method of regeneration is that the incipient shoot already has been differentiated, only elongation and root differentiation are needed to create a full plant. In the other hand, non-meristematic tissues should undergo developmental changes that typically include the forming of calluses and their reorganization into plantlets. An increased mutation rate, especially in the case of polyploidization, is observed in many plant species if the intermediate callus is involved. In addition, meristem-derived plants are phenotypically homogeneous, demonstrating genetic stability.

2.8.5.2. Virus-Free Plants Regeneration through Callus Cultures

For several species, the regeneration of plants from tissue and cell cultures is now an established procedure. The amounts of viruses from systemically contaminated plants in cultured tissues are considered to be very poor, and often the virus can be entirely lost. In cultured potato cells,

Morel has demonstrated that the titres of potato viruses X and Y are less than in intact plants. Augier de Montagremier and Morel have observed that TMV-infected crown gall callus tissues have a virus titrate thirty to forty times less than intact plant of infected leaves. Kassanis has observed that the TMV amount in the infected tobacco crown-gall cultures is around one thirtieth of that observed in the sap of infected tobacco leaves. Even if at the time of culture initiation, virus levels are significantly high, they will drop markedly through the duration of the process in the culture, and even disappearing totally. Chandra and Hildebrandt have been researching individual callus cells collected from TMV-infected tobacco plants in microchambers using the phase contrast microscopy. During successive passages on culture media, they also observed that the number of cells containing inclusion bodies (an indicator of infection) decreases. Five out of a total of one hundred cells containing inclusion bodies form colonies containing viruses. From such contaminated colonies, both healthy and infected plants could be regenerated. Some evidence was provided by Hirth and Eebeurier studies for inoculated tobacco cultures made out of a network of infected cells among the healthy cells. Hansen and Hildebrandt tested the presence of viruses with indicator plants and observed that infectious viruses produce only around 40% of mechanically separated callus cells derived from infected plants. Likewise, Hirth and Durr have shown that 60-70% of the cells from cultures of tobacco tissue contaminated with TMV are virus-free. However, most of these healthy cells present in the infected cultures are vulnerable to TMV infection, although they are not contaminated during tissue development. Kassanis has shown that TMV spreads at the same pace through tobacco callus tissue as through tobacco leaves. Svoboda assumes that virus-free areas emerge because callus cell proliferation, especially in cytokinin-containing media, proceeds more rapidly than virus replication. Several workers have been regenerated from callus colonies a virus-free plants. Chandra and Hildebrandt have produced tobacco plants from contaminated tissue cultures that are virus-free. Pillai and Hildebrandt and Abo-E1 Nil and Hildebrandt developed virus-symptomless plants derived from stem-tips and anthers from the callus of *Pelargoniurn hortorum*. Simonsen and Hildebrandt also create virus-free Gladius's plants with corms originating from cornel stem-tips from tissue cultures.

The aforementioned examples illustrate that viruses invade not all cells produced from infected culture tissues. Therefore, it should be possible to revive plants immune to viruses from a partially infected callus. While callus tissues appear to be a promising source of virus-free plants, we should be mindful that in callus cultures, chromosome abnormalities also occur. This can become a big limitation for the creation of callus culture vs. meristem cultures of virus-free plants.(Wang & Hu, 1980).

2.8.6. Nutrient Supplements

Against viruses it is not effective to add nutritional supplements to the plants or to control virus disease from the soil. While the treatment can mask the symptoms of the disease, the virus infection may not cure the vine; yet contaminated vines will also act as a virus vector to propagate to other vines. There is no empirical data to show that this therapy reverses the adverse consequences of virus transmission.

3. MATERIALS AND METHODS

3.1. Samples Collection

Leaf samples of a symptomatic grapevine were collected from a farm in Hungary (Visz, Somogy): red leaves with red veins, red leaves with green veins, that may suggest and mark grapevine leafroll virus infection, and leaves with red blotches and red veins, which might indicate the presence of red blotch virus, which has not been described from Hungary before.

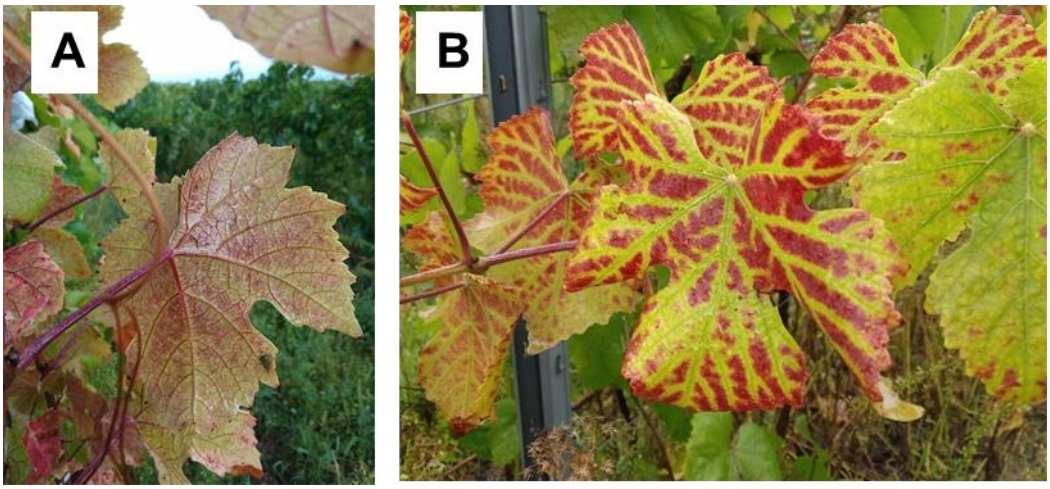

Figure 7: *Pictures of the surveyed Pinot noir grapevines showing A/ red blotch-like, B/ leafroll-like symptoms.*

Samples were collected from eleven different individual plants of the same row, only leaves were collected. All infected plants were from the same red berry, variety: Pinot Noir. The tested vines were grown in the same row of the symptomatic vineyard, showing either red blotch-like (1,2,3,5,7,8,10), or leafroll-like (4,11) symptoms “figure 7”, and from asymptomatic Pinot noir (6,9) and an additional asymptomatic green berries variety: Irsai Oliver (12) from the neighbouring row. Leaf samples were collected. The leaves were frozen at (-70) until RNA extraction.

The sampled plants showed a similar symptom to Red Blotch Virus, which was first identified in California. The disease is caused by a single-stranded circular DNA virus. Foliar symptoms typically begin appearing in mid-summer as irregular blotches on leaf blades at the base of infected grapevines. Over time, the blotches spread upward from the base to the top of the grapevine canopy.

3.2. RNA Extraction

Cetyltrimethylammonium bromide (CTAB) based technique was used to extract RNA from various sections of the collected sample, by the following protocol steps:

Heated at 65°C in a water bath the extraction buffer (prevent RNA degradation) which consist 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.

Mark 2 ml microcentrifuge tubes, then measure 850 µl extraction buffer in all microcentrifuge tubes and place them in a 65°C thermo-block.

Leaves samples of each plant (about 150/200 mg tissue) were homogenized using mortar with the addition of preheated extraction buffer and 17 µl ß- mercaptoethanol, the mixture was transferred back to the microcentrifuge tubes and vortexed thoroughly.

Then, samples were incubated at 65°C in a water bath for 10 minutes, during the incubation vortexed them at least once. After this, 850 µl of chloroform-isoamyl alcohol was applied to the samples accompanied for a few times by tube inversion. At 4 °C, the tubes were centrifuged for 10 minutes at 10,000 rpm.

The solution's supernatant (upper phase) was shifted to newly labelled tubes that already held 800 µl chloroform-isoamyl alcohol and centrifuged at 4 °C for 10 minutes at 10,000 rpm. New 1, 5 ml microcentrifuge tubes in which 250 µl 9 M LiCl were measured were labelled, Supernatant was transferred to fresh LiCl tubes after centrifugation accompanied by a quick inversion of the tubes. Tubes were then incubated on ice for 30 minutes.

At 4 °C, the samples were centrifuged once more for 20 minutes at 13,000 rpm. The supernatant portion of the solution was discarded.

The pellet was resuspended in 450 µl SSTE buffer preheated to 65°C. SSTE composed of (1 M NaCl, 0.5% SDS, 10 mM Tris base at 8.0 pH and 1 mM EDTA), followed by vortexing and equal volume 450µl of chloroform-isoamyl alcohol was added to the solution. Followed by centrifuging at 10.000 rpm for 10 minutes at 4°C.

Meanwhile, a new 1.5 ml tubes were labelled, containing 280 µl isopropanol and 30 µl 4 M Na acetate. The supernatant of the solution was transferred to them, invert the tubes and incubated them at room temperature for 5 to 10 minutes.

Centrifuge at 13.000 rpm for 20 minutes at 4°C the tubes. The supernatant part was discarded, and pellets were washed with 1 ml 70% cold ethanol followed with centrifuging at 13.000 rpm for 5 minutes at 4°C or room temperature and then, dried for 10 minutes in speed vac the tubes after removing the supernatant. The pellet was resuspended in 25 µl sterile water and vortexed gently.

Examination of the RNA product 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. That was achieved in the following actions: 3 µl of the extracted RNA were mixed with 5 µl FDE loading dye and 2 µl sterile water, after that denatured at 65°C for 5 min to finally run 10 µl from each RNA sample on 1.2% agarose gel.

Quantification of each sample using Nanodrop

3.3. Bioinformatics

Two small RNA sequencing libraries were prepared, 167-RB (symptoms look like Red Bloch), prepared from plants 1 and 10 and 168-LR (symptoms look like leaf-roll disease), prepared from plant 4 and 11. The libraries were sequenced at Illumina platform. The results (sequenced reads) were analysed using Qiagen CLC Genomic workbench. The reads were trimmed, both redundant and non-redundant list of sequences were prepared. The latter was used for contig building. Virus diagnostics were done by BLAST search of assembled contigs using all plant hosted viruses in the NCBI. The result list was ordered according to their lowest E-value. The reads (both redundant and non-redundant) were mapped to the GRBaV reference genome, and for other viruses which were present according to the analysis. AS a result, we got the list of the presenting viruses and viroids, which were organized according to their E-value (The lower the E-value is the higher the assurance of the presence of the virus or viroid). We set e-value lower than 10-5 as a threshold of virus presence and reorganized the list including the number of how many virus specific contigs were identified.

Using CLC Genomics workbench 10.0 program the reads of both libraries were mapped to the reference genome of the identified pathogen using the bellow key steps in CLC Genomic workbench.:

1- Choose ToolBox then NGS Core tool.

2- Choose Maps read to reference.
3- It will open Box so Choose element (167-RB trimmal reads EX. ”library sequence”) from Select elements part then Next.
4- New Box – Reference – Choose (GLRav1 – NC – 016509 EX. ”Referance genome of the pathogen”).
5- Next then again Next – without changing the parameters.
6- From the Output options choose Create report and from result handling Save the Next and Finish.

Save the map by the following steps:

Click right (Mouse) the File – Export Graphics- Export visible area – Next – Make sure PdF - Finish

The same program was used to compare consensus sequences too following the steps below:

1- Tool Box choose NGS Core Tool and Extract consensus sequence.
2- New Box will show in Select elements choose what interest in and Next.
3- From Coverage handling choose Insert “N” ambiguity Symbols.
4- Next and Next again without changing the parameters the Finish.

Save the sequence:

1- From the Tool Box choose Export – for FASTA – New box appear – Fasta – Description – Export seq. and seq. list in fasta
2- Select – Next (Select elements) – Next without changing in parameters set- Finish and Save

   “N” means there is no small RNA seq.

   After that the Coverage (N) were calculated by the program CLC:

1- Choose ToolBox.
2- Classical seq. Analysis
3- General seq. Analysis
4- Create seq. Statistics.

Available primers designed for the detection of the particular viruses were investigated for their ability to recognize (and amplify) the region of the viral strains present in the samples, utilizing NCBI database track the below steps:

1. NCBI choose BLAST then Nucleotide BLAST.
2. Choose align two or more seq.
3. From program selection choose somewhat similar seq.
4. For the first field enter the sequence of the pathogen need to be checked with the compatibility of primers Ex. (Cons. – seq. of GLRaV-1)
5. Second field the primer sequence (I chose them from the "List of grapevine virus primers")- (Enter the forward and the reverse each one separately.)
6. Next clicking the BLAST chose.

The results showed that the available primers are able to amplify the viruses in the samples, why we used them in the following RT-PCR validation and did not designed new ones.

3.4. cDNA Synthesis and Quality control

3.4.1. cDNA Synthesis

The first strand cDNA synthesis was performed using the "Thermo Scientific Kit" with the maximum amount of RNA template. First Strand cDNA Synthesis Kit is a full framework for effective first strand cDNA synthesis from mRNA or total RNA templates. I followed the recommendation of the purchaser starting by adding 0.5 µl of Random Hexamer Primer, to the extracted RNA. (5.5 µl of template RNA of four samples (1 – 10 RB / 4 – 11 LR)) resulting the total volume 6 µl. It was required to set the mix chill on ice, and spin down. Incubation was performed for 5 minutes at 65 °C, and we used PCR to keep it constant. Then the reaction mixture was chilled and spinned for 5 minutes, allowing the primers to perfectly bind to the template. A reaction mixture (RT) was added to the previous RNA mix incorporating 2 µl of 5x Reaction buffer per sample, 1 µl of 10 mM dNTP, 0.5 µl Ribolock RNase inhibitor (which protects RNA templates from degradation) and 0.5 µl Reverse transcriptase (RT) per sample (total volume was at 4 µl/sample). The 4 µl reaction mixture was added to the 6 µl of RNA template and primers before the incubation of the mixture was started and carried out as the Table 2 below:

Table 2: *Protocol for cDNA synthesis*

Procedure	**Temperature**	**Time**
Incubation	25 °C	5 minutes
	42 °C	60 minutes
	45 °C	15 minutes
	70 °C	5 minutes

The reverse transcription reaction product was used directly in PCR applications or stored at -20 °C for longer time

3.4.2. cDNA Quality Check

The quality of the cDNA synthesis was checked with a PCR reaction, amplifying a part of the host actin mRNA 'actin test,' using the "Phire Hot start DNA polymerase", what we called performing Actin test. This was initiated by diluting the generated cDNA 10 times (10xRT), then proceeded to a gentle vortex. The preparation of the reaction mixture was made up of the various components listed in the table 3 below.:

Table 3: *Quantity of reaction components of the cDNA control PCR mixture*.

Component	**Quantity per sample**
MQ (Sterile water)	9.4 µl
5x Phire Reaction Buffer	3 µl
Primer A (Vv actin 601 s)	0.75 µl
Primer B (Vv actin 1200 as)	0.75 µl
10 mM dNTP	0.3 µl
Phire Hot Start DNA Polymerase	0.3 µl
Template 10x RT	0.5 µl

The total volume per sample 15 µl. The number of samples 6 which four samples (1 – 4 – 10 – 11) and two samples (Negative – Positive control).

The PCR program was conducted using the following steps-Table 4:

Table 4: *PCR amplification protocol for actin test.*

Stage	Temperature °C	Time	Number of Cycle
Initial Denaturation	98	30 seconds	1
Denaturation	98	10 seconds	35X
Annealing	55	10 seconds	
Extension	72	20 seconds	
Final extension	72	1 minute	1
Hold	4	∞	-

Gel electrophoresis was performed on 1.2 per cent agarose gel to visualize the cDNA product with the load of 3 µl of the marker and 5 µl sample (to which we added 1vl DNA dye) To check the validity of the cDNA synthesis, an estimated 600bp PCR product should be visible. The voltage was 115V for 40 minutes.

3.5. Virus detection by PCR

In order to identify numerous grapevine viruses in the collected rootstock plants, RT-PCR reaction from the previously synthesized cDNA were conducted utilizing two different DNA polymerases to ensure that it is achieved correctly. The process was done by adding the formed cDNA to the mixture of PCR reagents, virus-specific primers as a single pair (forward and reverse) to the PCR reaction mix. Primer pairs for the detection of grapevine viruses using RT-PCR are summarised in Table 2.

3.5.1. Primers

Table 5: *List of virus-specific primers used for RT-PCR diagnostics.*

Virus	Primer name	Primer sequence (5'-3')	Function of the amplified region	Reference
GVA	GVA6591F GVA6862R	GAGGTAGATATAGTAGGACCTA TCGAACATAACCTGTGGCTC	coat protein	Goszczynski and Jooste, 2003
	GVA C FW GVA C REV	TGACCAGCCTGCTGTCTCTA TCGAACATAACCTGTGGCTC		

HSVd	HSVd-F HSVd-R	CTGGGGAATTCTCGAGTTGCC AGGGGCTCAAGAGAGGATCCG	genomic RNA	Farkas et al., 1999
GPGV	GPG6609F GPG7020R	GAGATCAACAGTCAGGAGAG GACTTCTGGTGCCTTATCAC	coat protein	Glasa et al., 2014
GYSVd 1	GYSVd1-F GYSVd1-R	TCACCTCGGAAGGCCGCCGCGG GTGAAACCACAGGAACCACAGG	genomic RNA	Czotter et al, 2018
GLRaV 1	GLRaV1 10372F GLRaV1 11404R	GCTCTCATAAACGAACCAACGTC CATGTAACTCAGAGAACATATCG	HSP70	Czotter et al, 2018
GVB	GVB F GVB R	GTGCTAAGAACGTCTTCACAGC ATCAGCAAACACGCTTGAACCG		
GRSPaV	RSPaV FW RSPaV REV	GGGTGGGATGTAGTAACTTTTGA GCAAGTGAAATGAAAGCATCACT	replicase	Gambino and Gribaudo, 2006
GFkV 1	GFkV1 F GFkV1 R	GGTCCTCGGCCCAGTGAAAAAGTA GGCCAGGTTGTAGTCGGTGTTGTC	replicase	Czotter et al, 2018
GSYV 1	SY5922F SY6295R	CCAATGGGTCGCACTTGTTG ACTTCATGGTGGTGCCGGTG	coat protein	Glasa et al., 201
GVT	GVT7630F GVT8534R	GTGTGGTCCTCGTTAGGTGC CGGCAAGAGTTCCAACTAGC	coat protein	Glasa et al., 2018
GLRaV 3	GLRaV3 FW GLRaV3 REV	TACGTTAAGGACGGGACACAGG TGCGGCATTAATCTTCATTG	coat protein	Gambino and Gribaudo, 2006
GRBaV 1	GRBaV1535f GRBaV1535r	GAGACGTCGATCTGAGCGCGGAG GGTGAATTCGTTAAGACGTTGAAG		Al Rwahnih et al 2013

3.5.2. PCR mixture and reaction condition

The methodology was followed similarly to the Phire Hot Start DNA Polymerase control actin test protocol, where the produced cDNA was incorporated into the PCR reagent mixture listed in the Table 3, Rather than actin primers set, virus-specific primers (forwards and backwards) have been applied to the PCR master mix, 14.5 µl of the master mix was spread to 6 PCR tubes, each holding 0.5 µl of 10x diluted cDNA samples (Samples 1, 4, 10, 11 / Negative and Positive Control Samples) for Phire Hot Start DNA Polymerase as shown in table 6, while 19 µl of the master mix was spread to 6 PCR tubes, each holding 1 µl of 10x diluted cDNA samples for Q5 Enzyme, mentioned in table 7.

Table 6: *Quantity of reaction components of the cDNA control PCR mixture for PHS Enzyme.*

Component	**Quantity per sample**
MQ (Sterile water)	9.4 µl
5x Phire Reaction Buffer	3 µl
Primer Forward	0.75 µl
Primer Reverse	0.75 µl
10 mM dNTP	0.3 µl
Phire Hot Start DNA Polymerase	0.3 µl
Template 10x RT	0.5 µl

Table 7*: Quantity of reaction components of the cDNA control PCR mixture for Q5 Enzyme.*

Component	**Quantity per sample**
MQ (Sterile water)	12.4 µl
5x Reaction Buffer	4 µl
Primer Forward	1 µl
Primer Reverse	1 µl
10 mM dNTP	0.4 µl
Q5 DNA Polymerase	0.2 µl
Template 10x RT	1 µl

Table 8: *PCR Annealing temperature using PHS and Q5.*

Pathogen	**Annealing Temperature with Q5**	**Annealing Temperature with PHS**
GVA	55 °C	55 °C
GPGV	62 °C	62 °C
GLRaV-1	53 °C	55.5 °C
GVB		55 °C
GRSPaV-1	58.7 °C	55 °C
GFkV	64.8 °C	62 °C
GSYV-1		60 °C
HSVd		60 °C
GYSVd-1		54.5 °C

3.5.2.1 PCR Gradient mixture and reaction condition

Gradient of annealing temperature in the PCR protocol (gradient PCR) was used with Q5 DNA Polymerase to determine the right annealing temperature for GFKV and GRSPaV-1 – for which the PCR reagent mixture is listed in the Table 9. 50 µl mixture was divided into 5 tubes.

Table 9: Quantity of reaction components of the cDNA control PCR mixture for Q5 Enzyme.

Component	**Quantity per 5 samples**
MQ (Sterile water)	31 µl
5x Reaction Buffer	10 µl
Primer Forward	2.5 µl
Primer Reverse	2.5 µl
10 mM dNTP	1 µl
Q5 DNA Polymerase	0.5 µl
Template 10x RT	2.5 µl

The total volume per sample 10 µl. The number of samples 10 for the two viruses were placed into the PCR instrument, where 5 different annealing temperature was settled according to Table 11.

The PCR program was conducted using the following steps-Table 10:

Table 10: *PCR amplification protocol with Q5 DNA Polymerase.*

Stage	**Temperature °C**	**Time**	**Number of Cycle**
Initial Denaturation	98	30 seconds	1
Denaturation	98	10 seconds	40X
Annealing	55 - 67	20 seconds	
Extension	72	30 seconds	
Final extension	72	2 minutes	1
Hold	4	∞	-

Table 11: The annealing temperature range.

Pathogen	Annealing chose temperature °C
GFKV	55.3
	58.7
	61.7
	64.8
	67
GRSPaV-1	55
	56
	58.7
	61.7
	64.8

3.6. Electrophoresis - Agarose Gel

To examine PCR-amplified DNA fragments and identify the existence of the virus specific products agarose gel electrophoresis was used and allowed for an easy representation of the data.

3.6.1. Agarose Gel preparation

The preparation of the agarose gel starts with measuring 3.6 g of agarose adding it to 300 ml 1x TBE buffer (TBE contain Tris base, Boric acid and EDTA "Ethylenediaminetetraacetic acid"). The mixture was heated for 30 seconds then shake it gently, The previous step was repeated until we got a clear homogenous mixture has completely dissolved using the microwave. Next, 1 µl EtBr (Ethidium bromide – which bind to DNA by intercalated between two adenine–thymine base pairs. and allow the visualization of the DNA under ultraviolet (UV) light)) was added into well dissolved agarose mixture as a quick step to pour it immediately into gel platform placing the comb in the right position. The gel was let to set in room temperature for 10 to 15 minutes until it has completely solidified.

3.6.2. Setting up the gel with the samples for separation

Using the electrophoresis set, the process of setting up the gel was different in one step between the samples done with PHS DNA Polymerase and Q5 DNA Polymerase.

3.6.2.1. Setting up the gel for samples been done with PHS DNA polymerase

The agarose gel was placed into the electrophoresis gel tank, upon placing the gel the tank was filled with 1x TBE with almost full. The gel was ready to start loading the samples, starting with a molecular weight ladder as a marker poured in the first well of the gel followed by the samples one after each another in the wells (3 µl ladder "Marker " was poured and 5 µl of each sample). Afterward, the gel was run at 115 – 130 Volt until a yellow dye line approximately got out the gel for 40 to 45 minutes.

3.6.2.2. Setting up the gel for samples been done with Q5 DNA polymerase

The agarose gel was placed into the electrophoresis gel tank and filled with 1x TBE as previous description in "3.6.2.a.". The first well was poured with ladder, DNA loading dye (Bromophenol blue) 5 µl was added to each sample. The samples were pipetted after each another in the wells (3 µl ladder and 10 µl of each sample were pipetted). The gel was run at 115 – 130 Volt for 45 minutes approximately.

3.6.3. Screening the Fragments

After electrophoresis the gel has illuminated by UV light to be able to see the EtBr stained DNA. For this we used "Bio-RAD chemidoc MP imaging system" which allowed us to take a picture of the gel.

4. RESULTS

RNA extraction and small RNA library preparation was carried out by my colleagues. The libraries were sequenced by UD-genomed. I joint to the project when the sequenced reads became available with the bioinformatics analysis of the data.

4.1. Results of bioinformatics analysis

To start the work with CLC program and we uploaded our data to and used Blast against the downloaded NCBI database which contained all of the reference genomes of known viruses and viroids, applying Lowest E – Value process. The results are summarized in Table 12.

Table 12: *Bioinformatic analysis, Using CLC program and NCBI Blast obtained for both libraries based on the lowest E-value and the remains of the confidential ones reorganized again based on the repeat time of the virus or viroid name.*

167-RB library	Virus list	Viroid list
	Grapevine leafroll-associated virus 1	Grapevine yellow speckle viroid 1
	Grapevine virus A	Grapevine yellow speckle viroid 2
	Grapevine pinot gris virus	Hop stunt viroid
168-LR	Grapevine virus B	Grapevine yellow speckle viroid 1
	Grapevine virus A	Grapevine yellow speckle viroid 2
	Grapevine leafroll-associated virus 1	Hop stunt viroid
	Grapevine fleck virus	

In parallel we used an alternative method, to map the small RNA reads to the known sequence of grapevine viruses to see how equally their genome is covered by small RNA reads. We have especially done this analysis for GRBaV because the previous method (BLAST of contigs) did not give us any hit for this virus. This showed a negative result about the presence of GRBaV in both libraries as it is shown in figures 8 and 9.

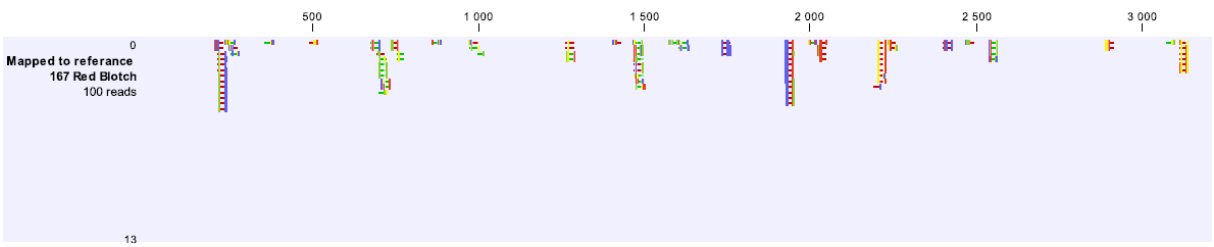

Figure 8: *167-RB library mapped to Red Blotch Virus.*

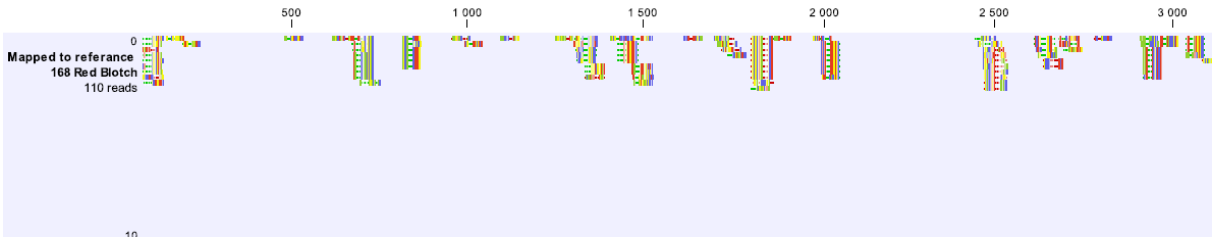

Figure 9:*168-LR library mapped to Red Blotch Virus (The rest maps of other pathogens can be found in the appendix 1.*

While we got very low amount of reads and low coverage for GRBaV, we identified several virus specific reads for other viruses Table 13 shows the reads number of hits for the other viruses.

Table 13: *The hit percent of the pathogen listed in "table 12" mapped to their reference genome.*

Virus	167-RB Reads	168-LR Reads	Viroid	167-RB Reads	168-LR Reads
Grapevine Red Blotch Virus	100 r	110 r	Grapevine yellow speckle viroid 1	9 999 r	6 405 r
Grapevine leafroll associated virus 1	76 631 r	156 117 r	Grapevine yellow speckle viroid 2	6 632 r	5 336 r
Grapevine Pinot gris virus	2 584 r	1 723 r	Hop stunt viroid	49 791 r	40 560 r
Grapevine Rupestris stem pitting associated virus – 1	2 038 r	3 051 r			
Grapevine Virus A	9 932 r	7 559 r			
Grapevine Virus B	766 r	9 797 r			
Grapevine fleck virus	184 r	8 163 r			

We also generated a number (in %) for coverage, where we counted positions of the small RNA reads contained information (compared to the presence of N-s, containing no sequence information for the position). in table 14:

Table 14: *detailed table illustrates the viruses and viroids coverage and other data.*

		viruses								viroids			
		GRBV	GLRaV-1	GVA	GVB	GFkV	GRSPaV	GPGV		HSVd	GYSPVd-1	GYSPVd-2	
RB library	contig	0	100	14	0	0	0	1		4	4	1	
	non-redundant reads	40	12900	1779	190	70	881	988		2639	958	86	
	redundant reads	100	76631	9932	766	184	3051	2584		49791	9999	6632	13.48
	redundant reads*	7	5685	737	57	14	226	192		3694	742	492	
	coverage	16.9	76.9	47.3	24.2	18.5	70.6	78.9		100.0	86.2	69.6	
	RT-PCR	0/2	1/2	1/2	0/2	1/2	2/2	2/2		yes	yes		
LR library	contig	0	179	24	1	2	0	0		5	4	1	
	non-redundant reads	40	25326	1772	171	1686	651	626		2329	485	251	
	redundant reads	110	156117	7559	9797	8163	2038	1723		40560	6405	5336	15.75
	redundant reads*	7	9912	480	622	518	129	109		2575	407	339	
	coverage	0.0	98.1	76.0	36.7	94.3	76.3	94.0		100.0	100.0	95.7	
	RT-PCR	No	yes	yes	yes	yes	yes	yes		yes	yes		
	*normalized to read/1 million sequenced reads												

4.2. cDNA quality check

In order to screen both libraries for the existence of viruses, we had to make sure that we got the proper and a good cDNA template. cDNA was synthetized as the protocol "3.4.2." were mentioned above using random hexamer primer with the maximum amount of RNA templates and no nuclease free water have been used. The actin test was carried out with "sense – antisense" actin primers which shows that our cDNAs synthesized were proper and good amplified products as it shown in figure 10. Positive and negative control indicated that our process was convenient.

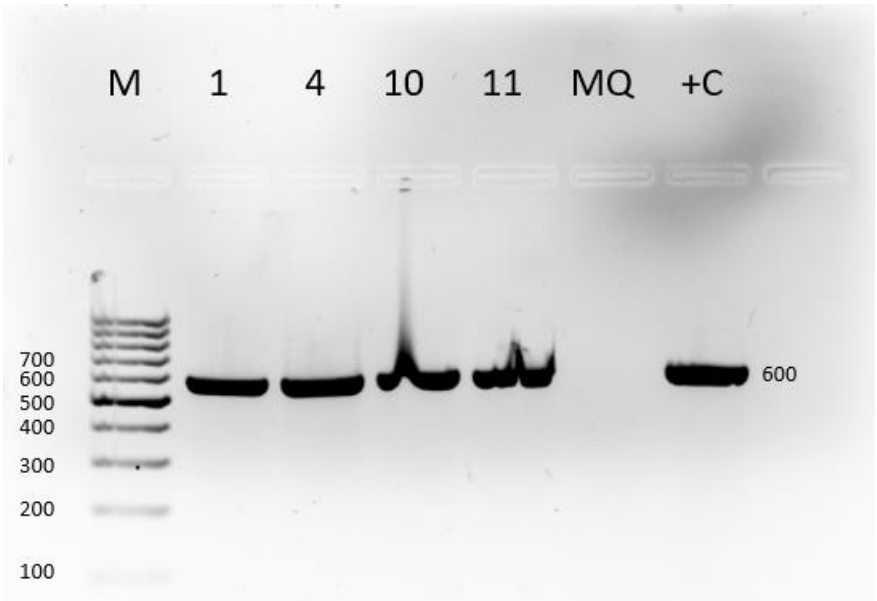

Figure 10: *Actin test result – (M) marker, sample 1 from library RB, sample 4 from library LR, sample 10 from library RB, sample 11 from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

4.3. PCR Gradient Results

Gradient PCR is a technique which uses the least number of steps to empirically determine an optimal annealing temperature.(Prezioso & Jahns, 2000). In simplified way, the gradient PCR is not a kind of real PCR, it is an alteration of the conventional PCR that facilitates the optimization of the PCR response by determining the exact temperature of the annealing. The main purpose of PCR outcome is to effectively amplify the DNA with high accuracy and high yield. (Chauhan, 2019). The thermocycler 's gradient method enables us to evaluate a number of temperatures in a single experiment. The optimum annealing temperature of the amplification must be set to preserve it high enough to reduce the number of non-specific products but not reducing the yield of the target product. Setting a variety of annealing temperature as described in table 8 to identify the optimization temperature using Q5 polymerase enzyme for both GFKV (GFKV1 F - GFKV1 R) primers and GRSPaV-1 (RSPaV FW - RSPaV REV) primers. As it shown in figure 11 optimized annealing temperature for GFkV was 64.8°C, for GRSPaV-1 was 58.7°C which been used later for RT-PCR validation.

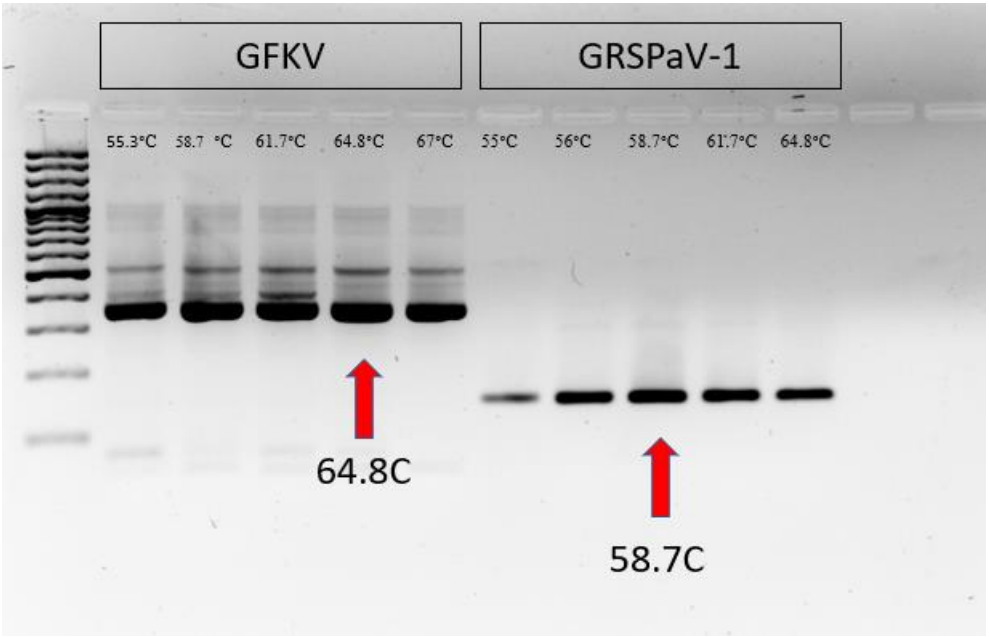

Figure 11: *PCR Gradient, PCR Gradient with the use of Q5 Polymerase enzyme to obtain the optimization annealing temperature for both GFkV and GRSPaV – 1.*

4.4. Validation of the results of small RNA HTS (presence of viruses and Viroids) using RT-PCR

RT-PCR was carried out using specific virus primers to identify the presence of the viruses and viroids. RT-PCR reactions were done not only to validate the presence of viruses listed in table

12 but also for the ones which, according to our knowledge, are widely spread in Hungary in order to ensure that they are absent. As a positive control we used cDNAs from previous RTs proved to be positive for the investigated virus, viroid`s from our group work.

4.4.1. Grapevine Leafroll – associated virus 1 (GLRaV – 1)

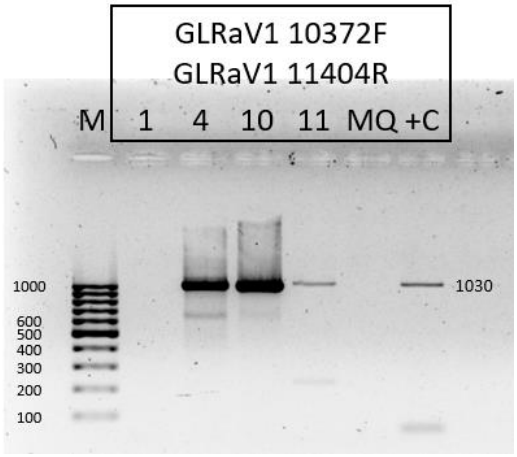

***Figure 12**: RT-PCR – GLRaV – 1, GLRaV-1 result using PHS polymerase – (M) marker, sample 1 from library RB, sample 4 from library LR, sample 10 from library RB, sample 11 from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

Using specific primers as mentioned in table 5 the appearing bands should be around 1030 bp. The RT-PCR was performed with PHS polymerase showing a successful reaction result. The virus was indicated in both libraries (RB – LR) expressed within samples (4 – 10 – 11) unlike sample (1) which shows a negative result free of (GLRaV – 1), figure 12.

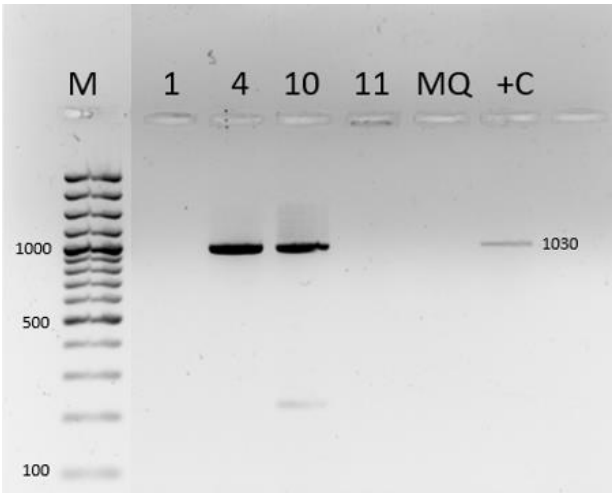

Figure 13: *RT-PCR – GLRaV – 1, GLRaV-1 result using Q5 polymerase – (M) marker, sample 1 from library RB, sample 4 from library LR, sample 10 from library RB, sample 11 from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

The use of Q5 Polymerase displays a positive result as PHS for samples (4 – 10) but in opposition to sample 11 from library LR which disappeared while using Q5 Polymerase giving a negative result due to its low concentration of the virus as it shown in figure 13.

4.4.2. Grapevine virus A (GVA)

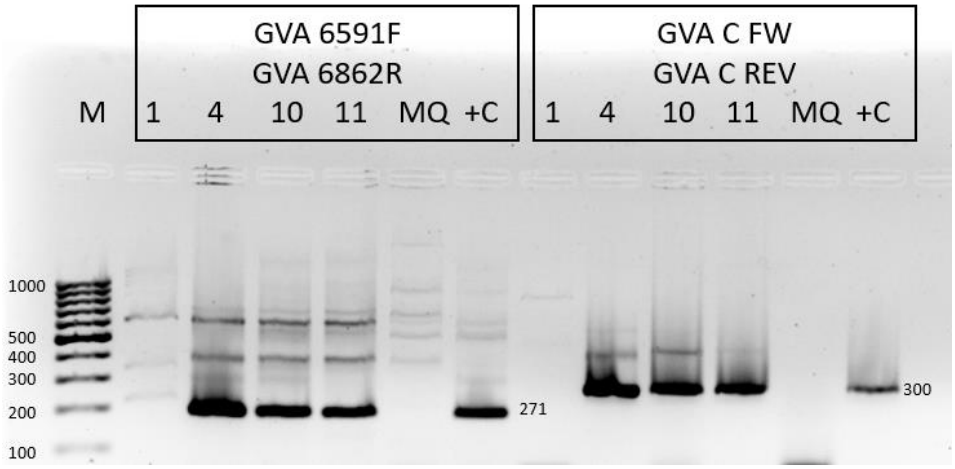

Figure 14*: RT-PCR – GVA, GVA result using PHS polymerase and two different primer pairs – (M) marker, sample 1 from library RB, sample 4 from library LR, sample 10 from library RB, sample 11 from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

RT-PCR reaction were handled with two specific primer pairs which was mentioned in table 5.

(F GVA6591F - R GVA6862R) primer shows a band around 271 bp. (GVA C FW- GVA C REV) primer shows a band around 300bp. Samples 1 to 4 were handled with the use of primer pairs (F GVA6591F - R GVA6862R), positive results, except for sample number 1, were captured in figure 14. Samples from 1 to 4 were done using primer pairs (GVA C FW- GVA C REV) also showed a positive result except for sample 1 which illustrated a negative result figure 14. RT-PCR reaction was performed with PHS polymerase.

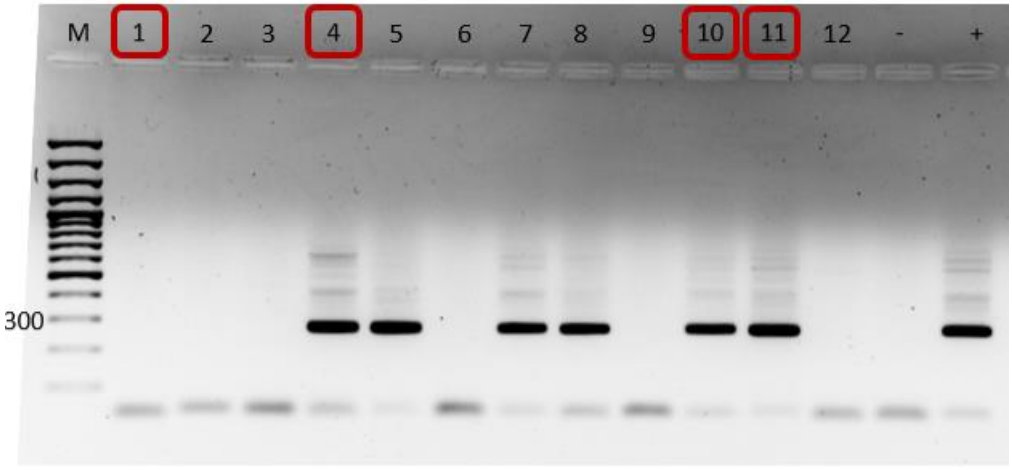

***Figure 15**: RT-PCR – GVA, GVA result using Q5 polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (-) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

Q5 Polymerase enzyme within RT-PCR reaction for GVA detection showed a positive result as PHS in samples (4 – 10 – 11), nevertheless a negative sample (1) from library RB "figure 15".

4.4.3. Grapevine pinot gris virus (GPGV)

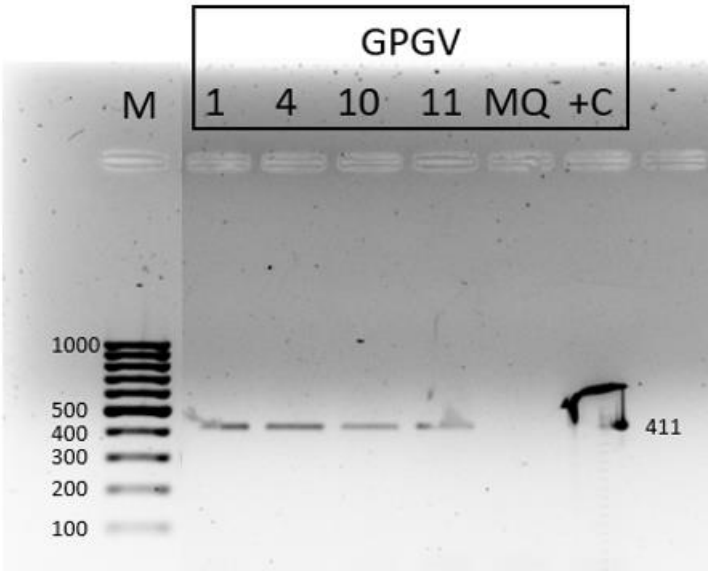

***Figure 16**: RT-PCR – GPGV, GPGV result using PHS polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

GPGV detection performed with PHS polymerase, gave a positive result in all samples “figure 16”. Using specific primers as mentioned in table 5 the bands should be around 411 bp.

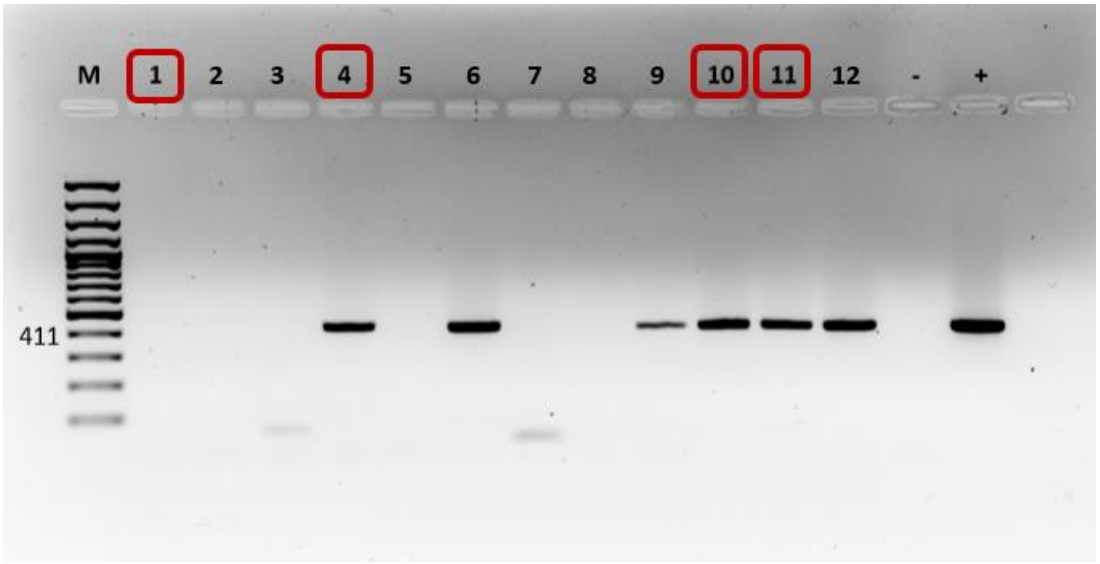

***Figure 17**: RT-PCR – GPGV, GPGV result using Q5 polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (-) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

GPGV validation with the use of Q5 Polymerase enzyme during RT-PCR reaction shown a positive result as RT-PCR using PHS Polymerase within samples (4 – 10 – 11), while sample (1) which gave a negative result from library RB, gave a positive result in PHS polymerase reaction, that means the sample may contain the virus at a low concentration “figure 17”.

4.4.4. Grapevine fleck virus (GFKV)

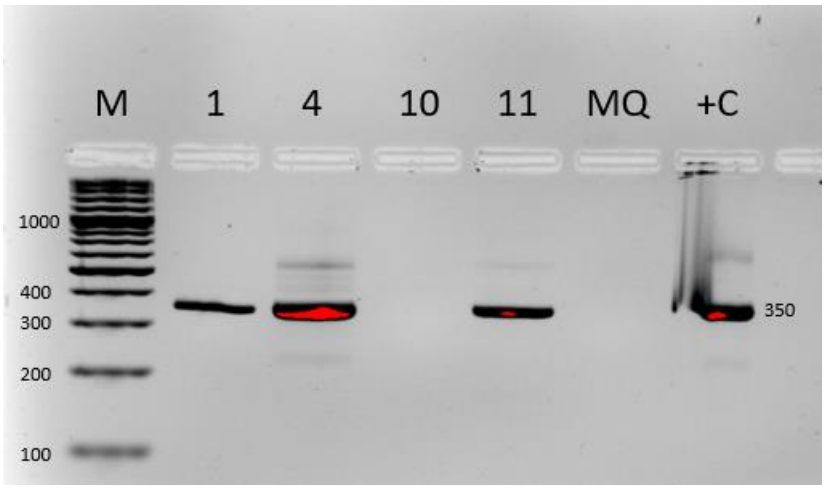

***Figure 18**: RT-PCR – GFkV, GFkV result using PHS polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

RT-PCR validation for GFkV using specific primers as mentioned in table 5 gave band around 350 bp. The reaction performed with PHS polymerase enzyme showing positive results in (1 – 4 – 11) samples, sample number (10) from library RB shows a negative result "figure 18".

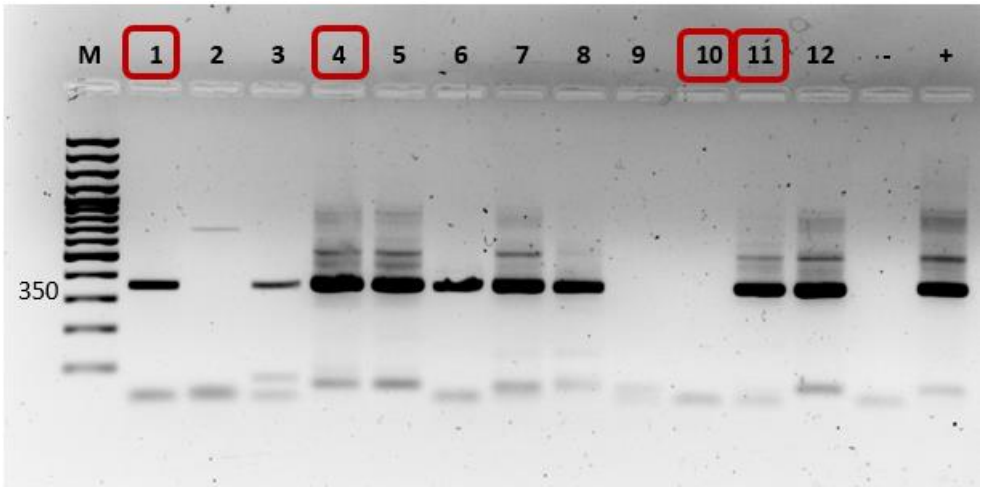

***Figure 19**: RT-PCR – GFkV, GFkV result using Q5 polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (-) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

To confirm the results of RT-PCR reaction using the PHS Polymerase, another reaction with Q5 Polymerase was conducted and that produced the same results "figure 19".

4.4.5. Grapevine virus B (GVB)

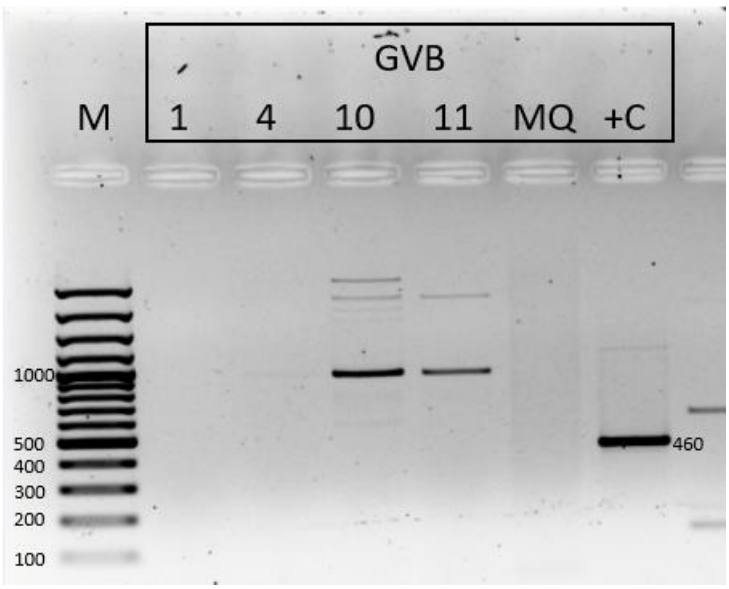

***Figure 20**: RT-PCR – GVB, GVB result using PHS polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

Using specific primers as mentioned in table 5, the outcome band is around 460 bp. The reaction with PHS polymerase proved a negative result in all samples besides the bioinformatic results, both libraries mapped to GVB reference genome presenting an unsuccessful result “figure 20”. Based on RT-PCR reaction with PHS polymerase results there were no need to apply the RT-PCR reaction with the use of Q5 polymerase enzyme.

4.4.6. Grapevine Rupestnis stem pitting associated virus – 1 (GRSPaV – 1)

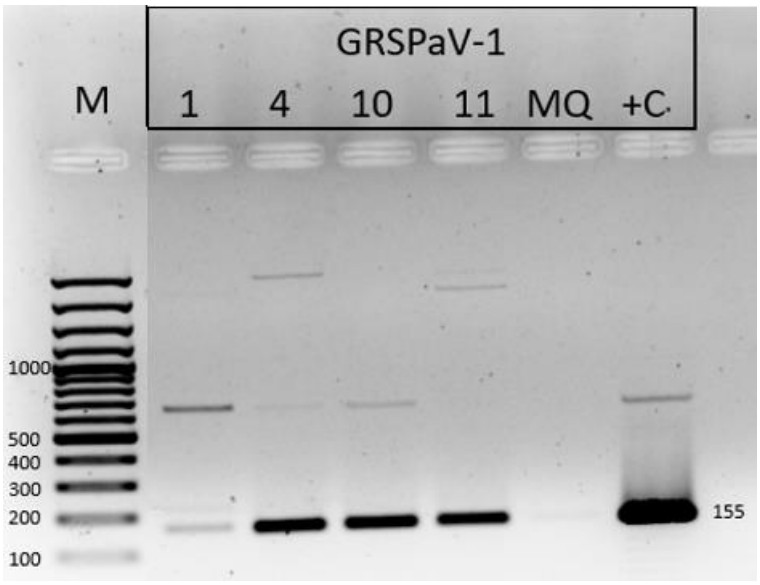

***Figure 21**: RT-PCR – GRSPaV – 1, GRSPaV – 1 result using PHS polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

The virus (GRSPaV – 1) not included in table 12 is widespread in Hungary which makes it interesting to analyse. Using specific primers as mentioned in table 5 the observe band around 155 bp. RT-PCR reaction with the use of PHS polymerase producing a positive result in all samples “figure 21”.

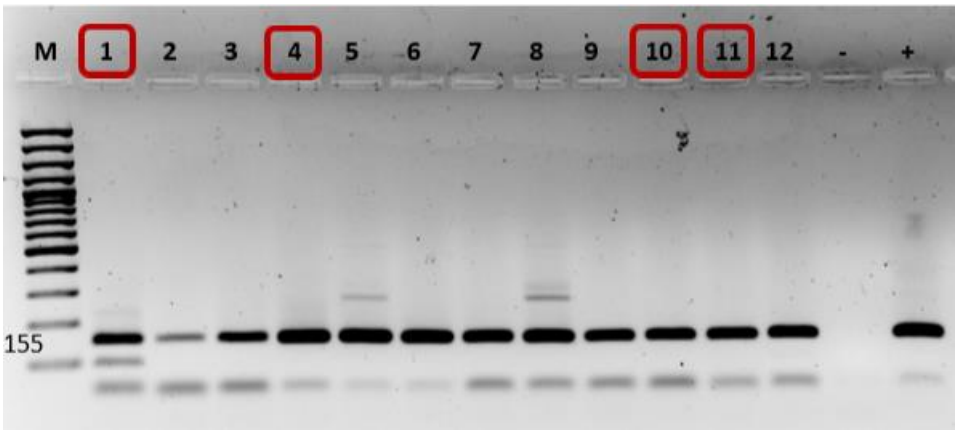

***Figure 22**: RT-PCR – GRSPaV – 1, GRSPaV – 1 result using Q5 polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (-) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

Q5 Polymerase was also applied, showing a positive result as PHS in samples (1 – 4 – 10 – 11) “figure 22”. The RT-PCR reaction with both PHS and Q5 polymerase enzymes ensure the virus existence.

4.4.7. Grapevine Virus T (GVT)

GVT is similar to GVA and GVB in a portion of its genome which drive our concerns to analyse it, although it is not present in the list table 12. Using specific primers as mentioned in table 5 the appearing bands should be around 904 bp as the positive control. The reaction performed with the use of PHS polymerase showed negative results in all “figure 23”.

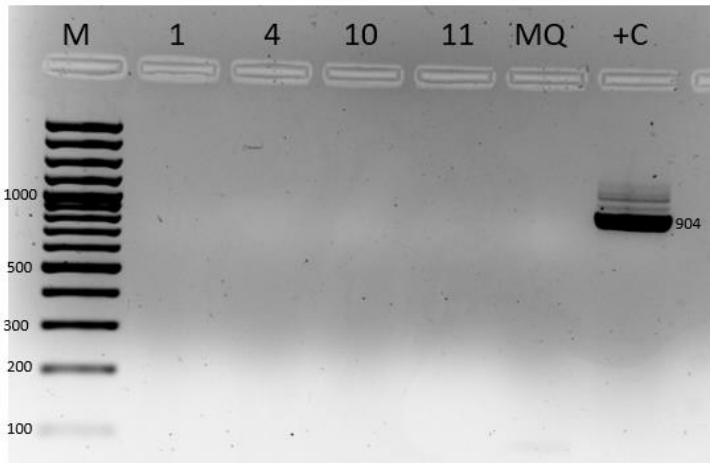

***Figure 23**: RT-PCR – GVT, GVT result using PHS polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

4.4.8. Grapevine Syrah Virus – 1 (GSYV-1)

Bioinformatic analyses did not indicate the virus, however it’s a common virus that draws our attention to evaluate its existence. With the use of specific primers as mentioned in table 5 the appearing band is around 373 bp, as it is in the positive control. The reaction was performed with PHS polymerase giving a negative result “figure 24”.

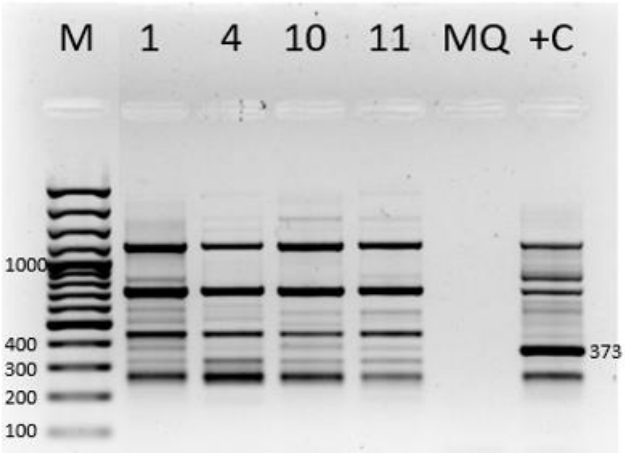

***Figure 24**: RT-PCR – GSYV – 1, GSYV-1 result using PHS polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

4.4.9. Grapevine Leafroll – associated virus 3 (GLRaV-3)

GLRaV – 3 genome has an identical portion to GLRaV – 1 which been detected in both libraries captured our attention to check it. Specific primers as mentioned in table 5 were used. The positive control band is around 336 bp. The reaction Processed with PHS polymerase showing a negative result "figure 25".

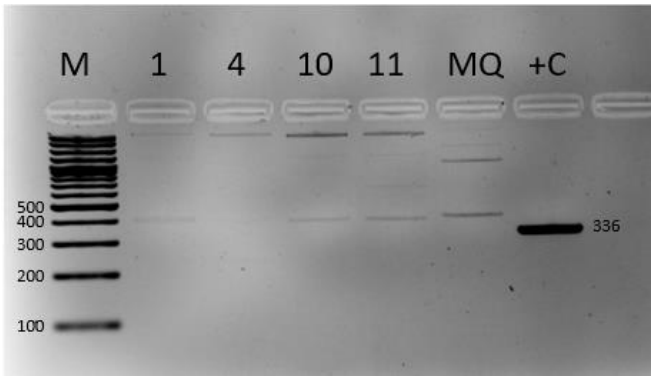

***Figure 25:** RT-PCR – GLRaV – 3, GLRaV-3 results using PHS polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

4.4.10. Grapevine yellow spekle viroid – 1 (GYSVd – 1)

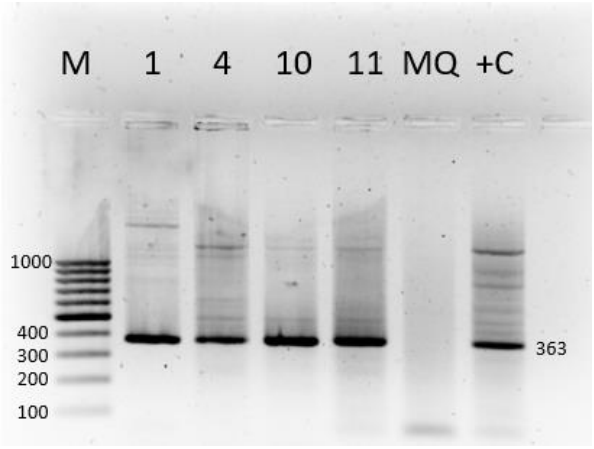

***Figure 26**: RT-PCR – GYSVd – 1, GYSVd - 1 results using PHS polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

Specific primers used as it is mentioned in table 5, producing bands around 363 bp. The reaction was processed with the use of PHS polymerase demonstrates a positive result in all samples "figure 26". Bioinformatic analysis shown the availably of the viroid in both libraries as the RT-PCR reaction.

4.4.11. Hop Stunt Viroid (HSVd)

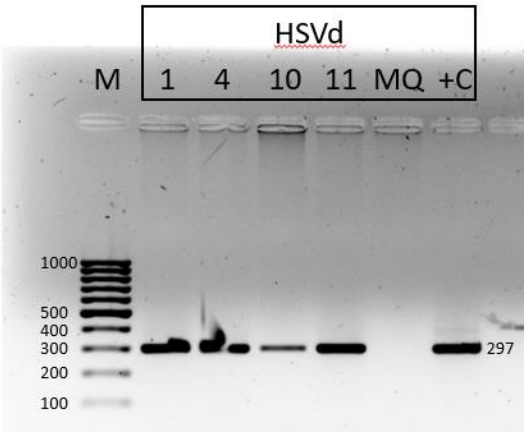

***Figure 27**: HSVd result using PHS polymerase – (M) marker, sample (1) from library RB, sample (4) from library LR, sample (10) from library RB, sample (11) from library LR, (MQ) negative control, (+) positive control from our group work a previous grapevine sample cDNA proved to be positive for the investigated virus.*

Using specific primers as mentioned in table 5 to validated HSVd, emerging bands around 297 bp. RT-PCR reaction with use of PHS polymerase display a positive result “figure 27”. HSVd viroid indicates its presence in both libraries even with the bioinformatic analysis.

5. CONCLUSIONS AND RECOMMENDATION

Results of the small RNA HTS show that several viruses: GLRaV-1, GVA, GFkV, GPGV and possibly GVB and viroids: HSVd, GYSVd-1 and 2 were present in the tested plants "Table 15", but neither GRBaV positive contig, nor reads mapped to the GRBaV genome were identified. Moreover, we could not get any product in the RT-PCR reaction using virus specific primers.

Table 15: *Summary of the bioinformatics analysis together with the RT-PCR validation. GRBaV: grapevine red blotch-associated virus. Numbers indicate PCR positive samples out of the 2 which served for mall RNA library preparation.*

	type of analysis	viruses							viroids	
		GRBV	**GLRaV-1**	**GVA**	**GVB**	**GFkV**	**GRSPaV**	**GPGV**	**HSVd**	**GYSPVd-1/2**
RB library	small RNA HTS	0	y	y	0	0	0	y	y	y
	RT-PCR	0/2	1/2	1/2	0/2	1/2	2/2	2/2	2/2	2/2
LR library	small RNA HTS	0	y	y	0	y	0	0	y	y
	RT-PCR	No	2/2	2/2	0/2	2/2	2/2	2/2	2/2	2/2

GLRaV-1: grapevine leafroll associated virus-1, GVA: grapevine virus A, GVB: grapevine virus B, GFKV: grapevine fleck virus, GRSPaV: grapevine rupestris-associated virus, GPGV: grapevine Pinot gris virus, HSVd: hop stunt viroid, GYSVd - 1 and 2: grapevine yellow speckled viroid 1 and 2.

We have found severe infection with GLRaV-1 and also the presence of several other viruses and viroids "Table 15". In case of GLRaV-1, GVA, GPGV and the viroids our RT-PCR results verified the result of the small RNA HTS "Figure 28". We could not prove the presence of GVB, but we have found only 1 GVB positive contig and less than 40% coverage of the genome, which indicate a false positive hit during the analysis. In RB library GFkV was not detected by small RNA HTS, but in RT-PCR one of the plants showed infection. RNA from the other plant could dilute the sample what was used for small RNA sequencing, why we failed to detect it by this method. For GRSPaV – 1 we found the same contradiction, but this is what we usually experience in case of this virus. There were very few GRSPaV – 1 derived small

RNA reads in the sample while the virus was present. One explanation of this can be that it was proved that this virus can have a positive effect on the grapevine physiology why defence mechanism against it could be suppressed during the evolution.

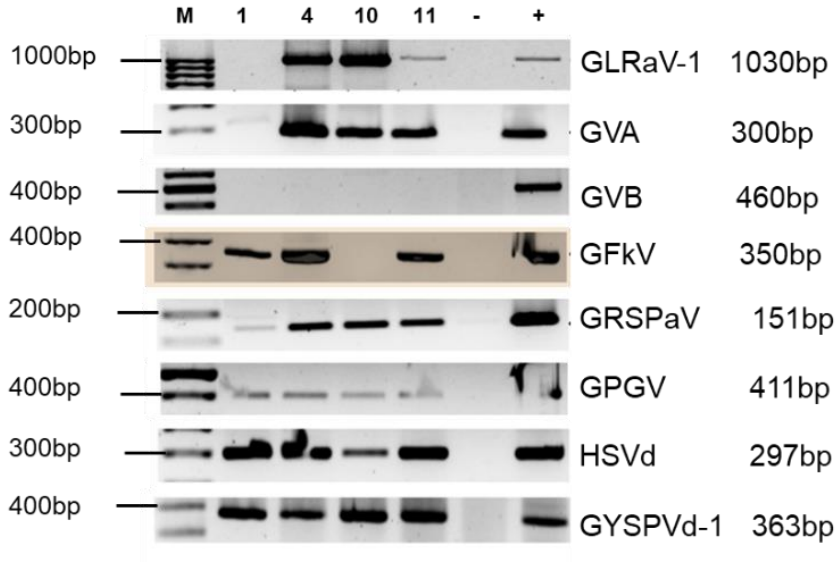

Figure 28: *RT-PCR analysis for testing the presence of different viruses in the four plants which small RNA was sequenced.*

In a conclusion the presence of several different viruses seemed random in the plants, thus we could not correlate any special combination with the appeared symptoms.

6. SUMMARY

Our results showed that although red blotch symptoms appeared, GRBaV was not identified in the investigated Hungarian vineyard. Although it seemed possible for us to detect the presence of several different viruses and viroids, we cannot make any hypothesis about their contribution in the observed symptom development. Distribution of the nutrient in the soil of this vineyard is very patchy why it is possible that shortage of some of them occurs quite randomly. Combination of these abiotic effects with the virus infections could lead finally or play role in the observed symptom development, but to find out the real causative agent we need further investigations. Moreover, these ambiguous results highlight the importance of the cooperation of classical and molecular virologist to reveal practical importance of the detected virus infections and explain or disclose possible causes of the emerging symptoms.

7. ACNOWLEDGEMENTS

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8. REFERENCES

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(https.3) https://viralzone.expasy.org/7316

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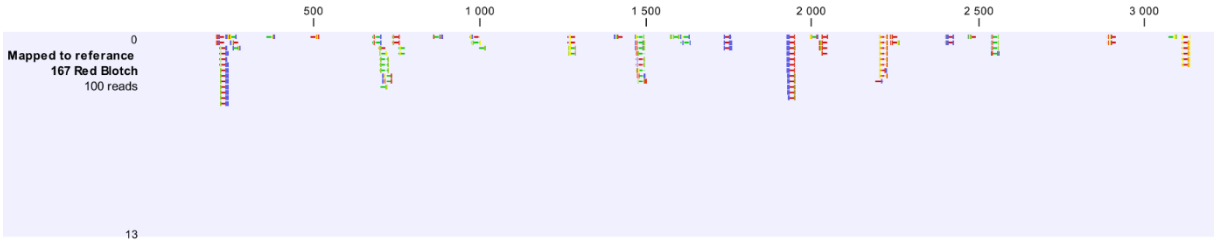
(https.5) https://www.virology.ws/2010/07/16/detection-of-antigens-or-antibodies-by-elisa/

APPENDICES

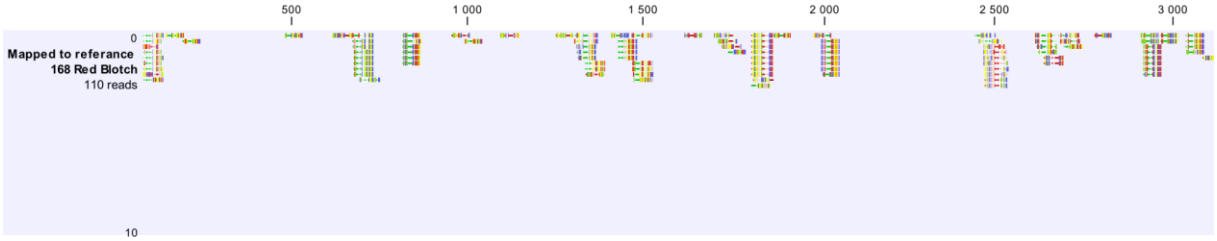
APPENDIX (1)

Graphs that shows the hit precent by reads of the viruses and viroids mapped to their reference genome for both libraries RB (Red Blotch) – LR (Leaf – Roll symptoms like).

GRBaV

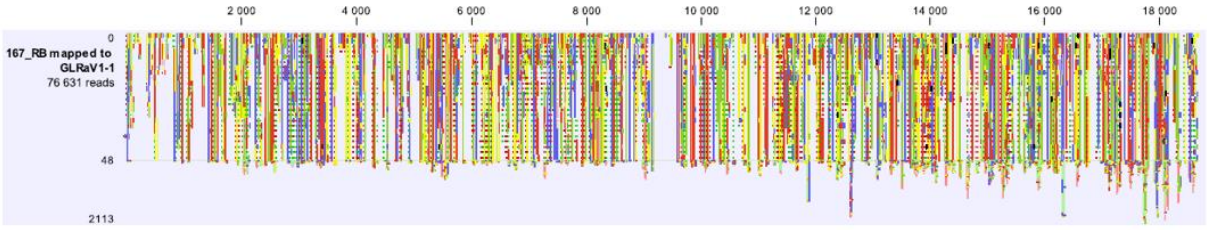


Graph 1: GRBaV reference genome mapped to RB library.

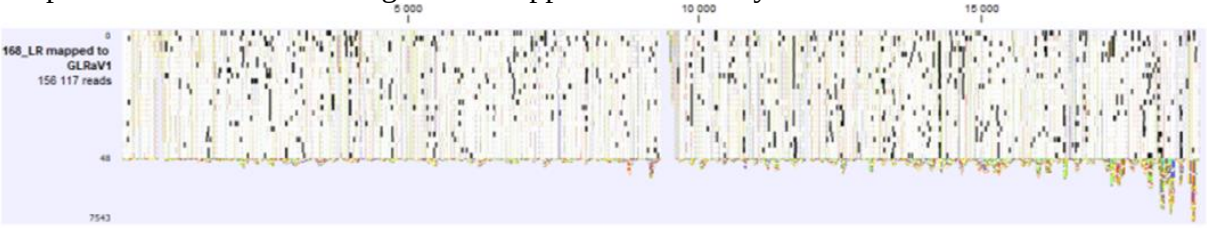


Graph 2: GRBaV reference genome mapped to LR library.

GLRaV-1



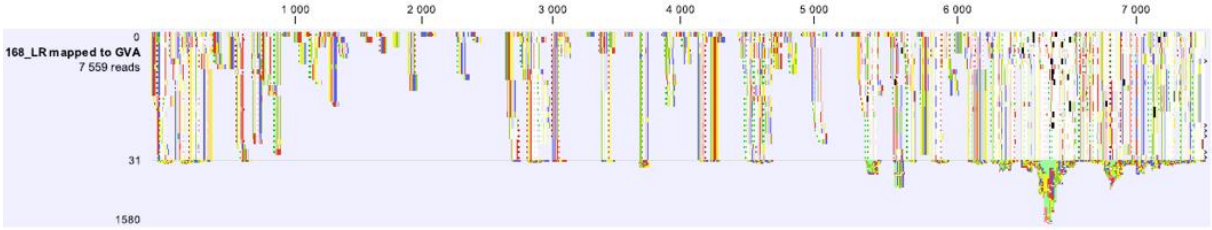
Graph 3: GLRaV-1 reference genome mapped to RB library.



Graph 4: GLRaV-1 reference genome mapped to LR library.

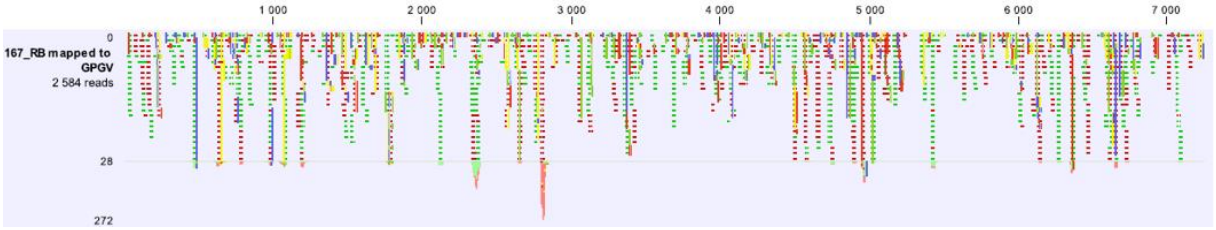
GVA

Graph 5: GVA reference genome mapped to RB library.

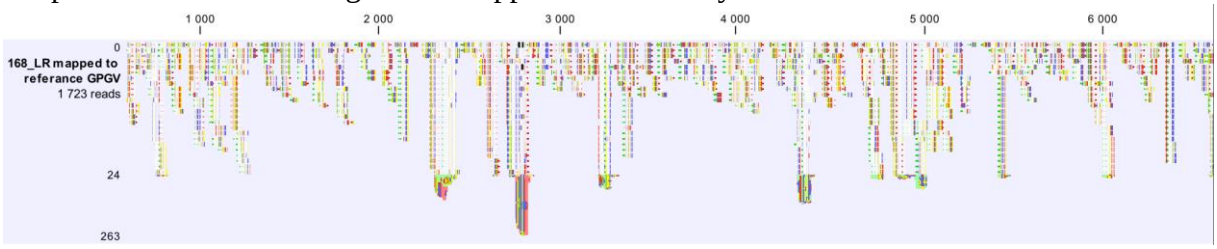


Graph 6: GVA reference genome mapped to LR library.

GPGV

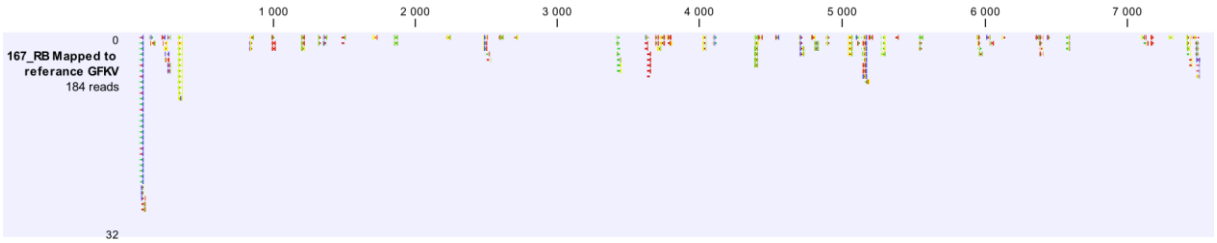


Graph 7: GPGV reference genome mapped to RB library.

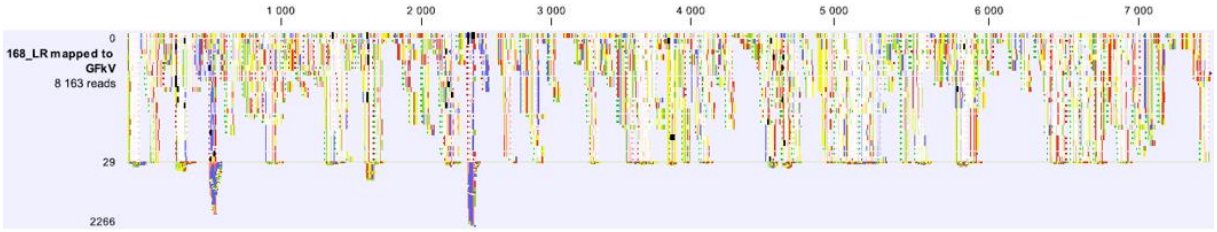


Graph 8: GPGV reference genome mapped to LR library.

GFkV

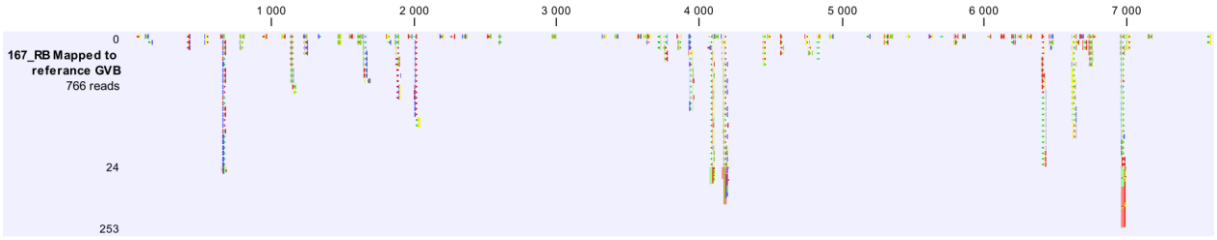


Graph 9: GFkV reference genome mapped to RB library.

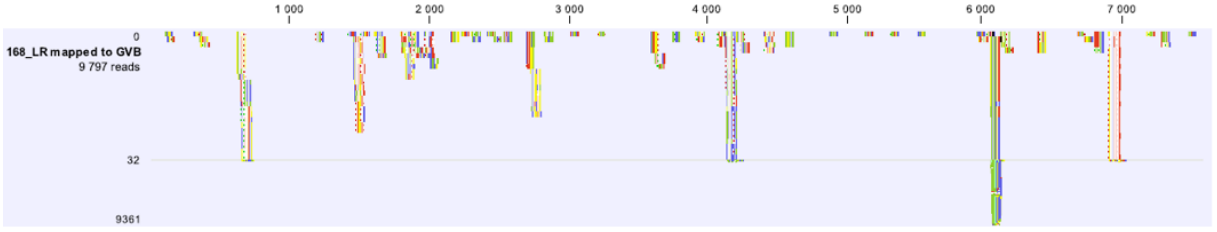


Graph 10: GFkV reference genome mapped to LR library.

GVB

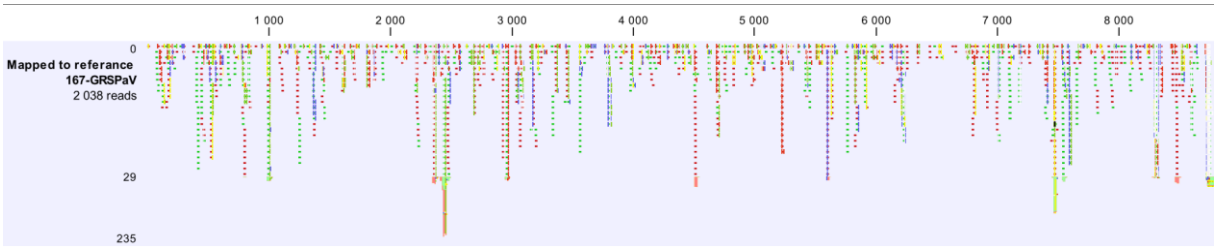


Graph 11: GVB reference genome mapped to RB library.

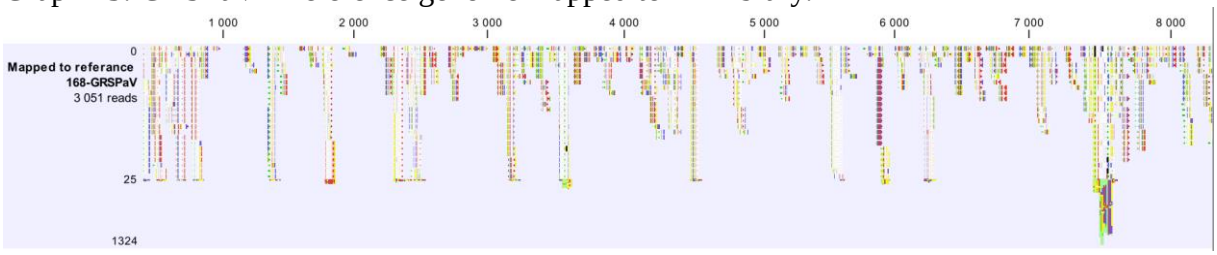


Graph 12: GVB reference genome mapped to LR library.

GRSPaV-1

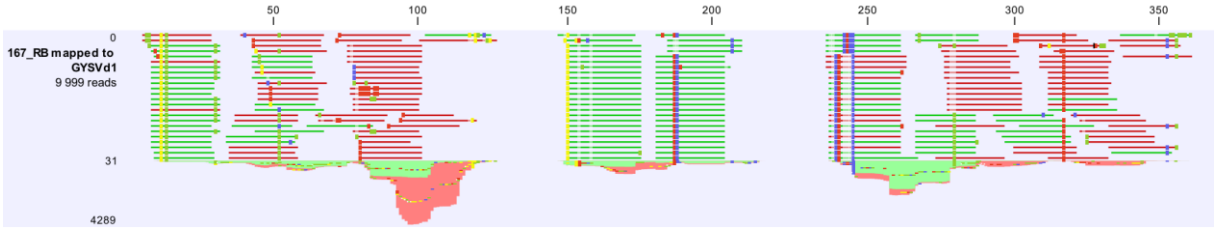


Graph 13: GRSPaV-1 reference genome mapped to RB library.



Graph 14: GRSPaV-1 reference genome mapped to LR library.

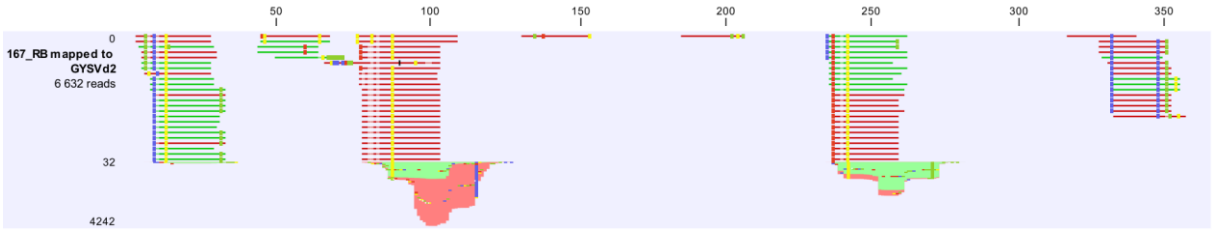
GYSVd – 1



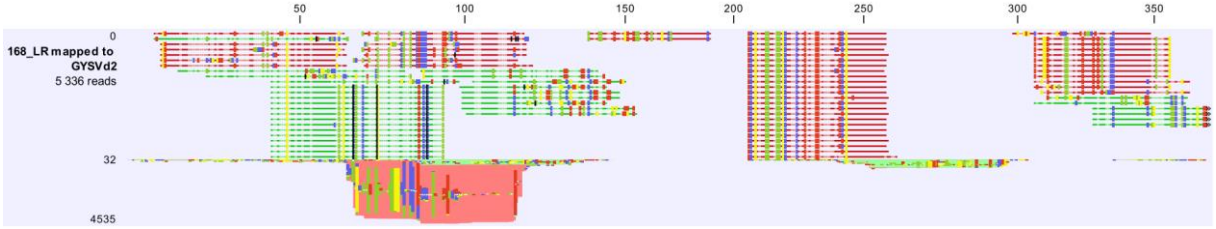
Graph 15: GYSVd-1 reference genome mapped to RB library.

Graph 16: GYSVd-1 reference genome mapped to LR library.

GYSVd – 2

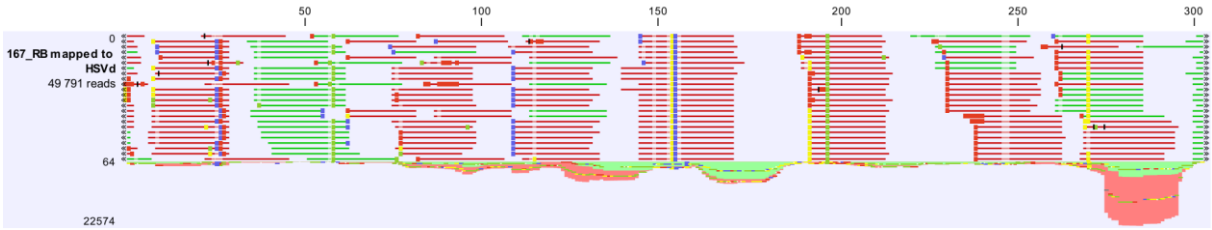


Graph 17: GYSVd-2 reference genome mapped to RB library.

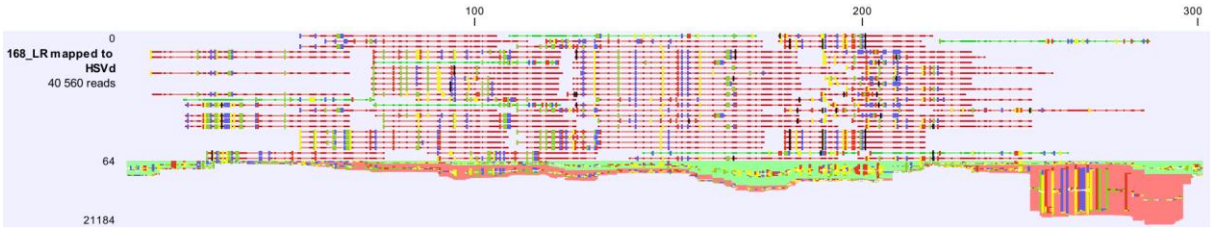


Graph 18: GYSVd-2 reference genome mapped to LR library.

HSVd



Graph 19: HSVd reference genome mapped to RB library.



Graph 20: HSVd reference genome mapped to LR library.

APPENDIX (2)

SAMPLES PHOTOS USED IN BOTH LIBRARIES

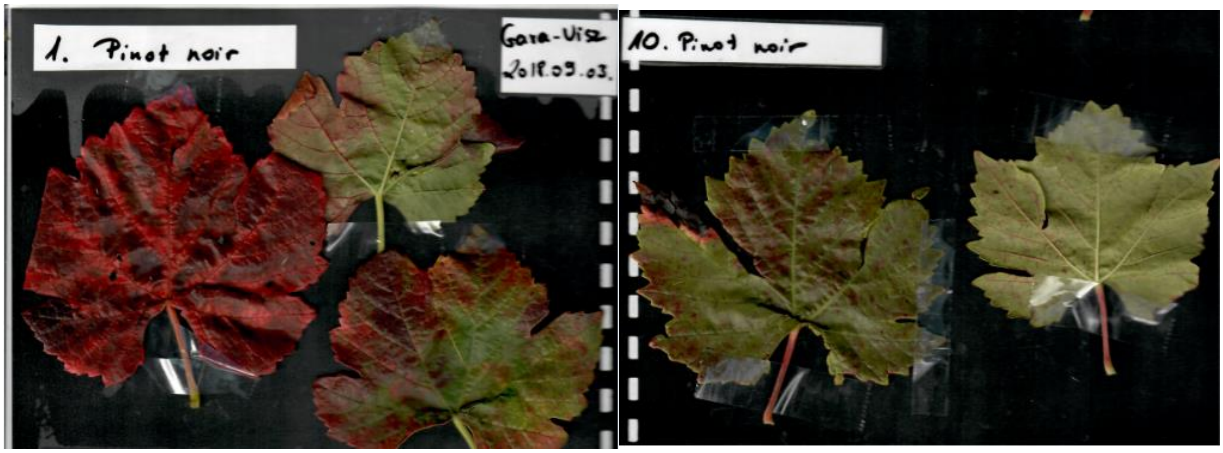


Figure 1: RB library from sample 1 and sample 10

Figure 2: LR library from sample 4 and sample 11

9. DECLARATION

9. DECLARATION

Signed below, …**Dana Khrais**…, student of the Faculty of Agricultural and Environmental Sciences, Szent István University, at the **MSc** Course of ……**Agricultural Biotechnology** …… declare that I have used the cited and quoted literature in accordance with the relevant legal and ethical rules. I understand that the one-page-summary of my thesis will be uploaded on the website of the Faculty/Institute/Course.

Confidential data are presented in the thesis: yes no

Gödöllő, 21..day ……Oct.……month.2022.year

……

Student's signature

As primary thesis adviser of the author of this thesis, I hereby declare that review of the thesis was done thoroughly; literature sources cited in the dissertation were used in accordance with the relevant legal and ethical rules. I hereby, approve the thesis for oral defense on Final Examination.

Confidential data are presented in the thesis: yes no

Gödöllő, 21..day ……Oct.……month.2022.year

……

Signature of the Primary Thesis Adviser