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Review

Methods for the Diagnosis of Grapevine Viral Infections: A Review

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Abstract: The yielding capacity of grapevine growth and the quality of the resulting product heavily depend on the health of the cultivated plants. The phytopathogens affecting the vineyards can cause a significant reduction in the yield and quality of the product. For this reason, it is extremely important to use diagnostic methods that make it possible to identify the pathogens, and to choose the correct method of plant protection. This review considers the main viral grapevine pathogens, and the existing methods of their diagnosis. The limitations of conventional diagnostic methods that are based either on the visual assessment of symptoms, or on bio-testing, are analyzed. A major focus is placed on two intensively developed approaches of diagnosis, molecular genetic and immunochemical methods. Applications of amplification techniques and DNA chips are presented, as well as opportunities for next-generation sequencing. A reduction of assay duration and labor intensity in combination with the assay shifts from specialized laboratories toward the places of sampling are considered as the main factors influencing the development of immunodiagnostic techniques. The potential place of diagnostic tests in vine-growing practices, and the requirements for their most efficient applications for early disease diagnosis is also discussed.

Keywords: grapevine; viral infections; phytopathogens; molecular diagnosis; immunodiagnosis

1. Introduction

Grapevine is an agricultural crop of major economic influence worldwide. It is noted [1] that the grape market is one of the most dynamic and fast-growing agricultural markets in recent years. Therefore, the timely monitoring of factors that can possibly have an adverse effect on plant growth and product quality, the causative agents of grapevine diseases in the first place, is of critical importance for grape production.

Grapes can be affected by various phytopathogens including fungi, bacteria, viruses, viroids, and phytoplasmas. Economically, the most important diseases among the fungal ones and their causative agents are: *Uncinula necator* Burill., various rots (*Botrytis cinerea* Pers., *Coniothyrium diplodiella* Sacc., *Aspergillus* spp., *Penicillium* spp., etc.), phomopsis blight (*Phomopsis viticola* Sacc.), fusarium diseases (*Fusarium sporotrichioides* Sherb, *F. chlamydosporum* Woll. et Rein., *F. moniliforme* Sheld., *F. oxysporum* Schlecht.), mildew (*Plasmopara viticola* Berl. et Toni.) and anthracnose (*Gloeosporium ampelophagum* Sacc.) [1]. A description of these diseases, and of the current practices for their control has recently been provided [2,3]. The bacterial pathogens affecting grapevine can cause necrotic burns,

spotting, inflorescence rot (*Pseudomonas syringae*), Pierce's disease *(Xylella fastidiosa)*, bacterial canker (*Agrobacterium tumefaciens, A. vitis)*, bacterial blight (*Xylophilus ampelinus*) etc. [4,5].

Viral disease agents are a special group of grapevine pathogens. It is reported that there are more viruses that affect grapevines than there are viral pathogens for other cultivated plants [6]. As of 2017 [7], there were more than 70 virus species belonging to 17 families and 27 genera that can cause grapevine diseases. This figure is constantly growing due to the use of next generation sequencing fir the identification of new pathogenic species (see Section 7) [8–11]. Disease symptoms caused by pathogenic grapevine viruses vary widely including reduced growth and development of the plant, leaf spotting, chlorosis, necrosis, streaking and ring-shaped spots on the leaves, leaf curling, leaf discoloration (yellowing or reddening), wrinkling, pitting or grooving of wood and swelling at the grafting site [7]. There is a decline in graft survival rate and the probability of the shoots taking root, for virus-infected plants. Viral infections start to have an adverse effect on the physiological activity of a grapevine long before the first symptoms appear. This means that the pathogens interfere directly with the plant's metabolism, causing a significant decline of photosynthesis in the leaves, and decreases in stomatal conductance and transpiration rate, as well as changes in the pigment concentration [12]. These changes, in turn, lead to delayed ripening, increased juice acidity and reduced yield [13]. The overall negative effect of viral infections results in a reduction of vineyard use time and a lower quality of berries. For instance, a leafroll infection alone can cause a ca. 15–20%, and sometimes even as high as 40%, drop in yield [14].

Viruses that cause grapevine diseases mostly have single-stranded RNA genomes, while some have a double-stranded RNA genome (Grapevine endophyte alphaendornavirus and Grapevine Cabernet Sauvignon reovirus). In recent years, grapevine pathogenic viruses with DNA genomes have also been found [15,16]. About half of the grapevine viral pathogens are associated with four main diseases: infectious degeneration and decline, rugose wood, leafroll and fleck [7] (Table 1).

Early identification of the infection's foci makes it possible to take measures to limit the damage of virus infection. These measures include the removal of affected vines, the limitation of the movement of agricultural machinery, the treatment of instruments and vector and weed control [17–19]. In addition, an infection is far less likely to develop if healthy planting material is used. In order to receive healthy planting material, or to make the existing material healthier, methods of meristem culture, thermotherapy and chemotherapy are used [20–23].

It is impossible to prevent the spread of viral diseases of plants without a reliable early diagnosis, including both infection confirmation and pathogen identification. Diagnostic methods have evolved significantly in recent times, and the often arbitrary visual symptom assessment is being replaced by modern bioanalytical tests that allow for reliable diagnosis. It is of critical importance to reduce test costs and duration in order to provide wide-scale diagnosis that can be included in comprehensive plant protection plans, making them available not only in laboratories but also in the field. Such a shift in testing sites would enable early information access, which would in turn ensure that necessary protective measures are taken sooner [24].

The evolution of grapevine viral disease diagnosis is described in works of Giovanni P. Martelli et al., including their recent monograph [7] by Meng et al. In this paper, however, it is deemed important to systematize the current state of affairs in this area by focusing on the available methods, and innovative technological solutions that have appeared only in recent years.

Hence, the present review provides information on the modern variety of grapevine viral pathogen detection techniques, the results of their application, as well as the kits and test systems available for the users.

Table 1. The main viruses causing diseases in grapes and infection symptoms.

Disease	Virus Name	Abbreviation	Systematic Position	Main Symptoms
Infectious degeneration and decline	*Grapevine fanleaf virus*	GFLV	*Secoviridae*, Nepovirus	Reduced vigour, increased sprout formation (branching), double nodes, short internodes, zigzag shoot growth; leaves are fan-shaped and asymmetrical, with veins grown together; chlorosis and yellow mottling of the leaves can also be present; small clusters, shot berries.
Rugose wood	*Grapevine virus A*	GVA	*Betaflexiviridae*, Vitivirus	Swollen and corky vine above the grafting site; different diameter of the scion and rootstock; pitted or grooved wood; leaves may curl; leaves may turn red or yellow.
	Grapevine virus B	GVB		
	Grapevine virus D	GVD		
	Grapevine Rupestris stem pitting-associated virus	GRSPaV	*Betaflexiviridae*, Foveavirus	
Leafroll	*Grapevine leafroll-associated virus 1*	GLRaV-1	*Closteroviridae*, Ampelovirus	Dark-skinned varieties show leaf reddening; in white varieties, leaves turn yellow or chlorotic; veins remain green; leaf blades curl.
	Grapevine leafroll-associated virus 3	GLRaV-3		
Grapevine fleck	*Grapevine fleck virus*	GFkV	*Tymoviridae*, Maculavirus	Clearing of the veinlets evolving into leaf mottling by the end of the vegetation period; shiny leaves; leaves may also curl upwards.
Red blotch	*Grapevine red blotch-associated virus*	GRBaV	*Geminiviridae*, Grablovirus	Leaf reddening in red-berried cultivars (chlorotic areas in white-berried cultivars) early in the growing season; delay of berries ripening.
Vein-clearing and vine decline	*Grapevine vein clearing virus*	GVCV	*Caulimoviridae*, Badnavirus	Clearing of secondary or tertiary veins on young leaves; mosaic and mottling on mature leaves; zigzagged and short internodes on a young shoot; brownish, deformed, irregularly shaped and small berries

2. Visual Diagnosis

Historically, visual diagnosis was the first and most used approach to diagnosing grapevine diseases. Such a diagnosis does not have significant material costs for special reactants. However, it is based only on the experience of an investigator, and it is therefore inevitably arbitrary, which can result in inaccurate pathogen identification.

Changes in grape leaf morphology and color are caused by a wide range of reasons. For example, leaf blade reddening can be indicative of either phytopathogens (viruses, phytoplasmas), or of mineral nutrition disorders, nutrient deficiencies, mechanical injuries (by pests such as mites, insects or rodents), or of plant exposure to pesticides [25]. Furthermore, leaf reddening and curling, for instance, can be caused by the *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 2* (GLRaV-2) and *Grapevine leafroll-associated virus 3* (GLRaV-3), a Grapevine flavescence dorée phytoplasma infection, or the *Grapevine red blotch-associated virus* (GRBaV) [25–27]. Moreover, the same virus can cause differing symptoms in different grape varieties. A GLRaV-3 infection in white-skinned varieties results in leaf blade yellowing, whereas in red-skinned varieties, it causes leaf reddening [27–29]. The severity of symptoms can also differ depending on the weather conditions, virus titre, strain aggressiveness, time of infection, disease duration, and the scion and rootstock combination [27]. Finally, in some cases, a viral infection can be asymptomatic [30]. This is why it is rather difficult to correctly detect and identify a virus on the basis of visual symptoms only, without the use of laboratory testing. Nevertheless, visual diagnosis remains a popular means of primary assessment for phytosanitary vineyard conditions.

3. Biological Testing

Biological testing implies the use of biomaterial from a grape plant to test for indicator plants, in which the infection symptoms are evident, clearly visible and specific. This method makes it possible to conclude if there is a defeat by a pathogen on a plant, determine a type of disease by getting clear symptoms (leaf curl or wood change). Two approaches are most often used to identify viral diseases of grapevine with biological testing, namely, inoculation with the juice of diseased plants, and inoculation of buds from a diseased plant [31–33].

On the one hand, viruses that can be transmitted mechanically, such as viruses belonging to the Nepovirus genus as well as some representatives of the Vitivirus and Closterovirus genera [34–36], are detected by the inoculation of indicator herbage plants with the sap of a sick tested plant. *Chenopodium quinoa*, *Ch. amaranticolor*, *Cucumis sativus*, *Nicotiana occidentalis*, *N. tabacum* and *N. benthamiana* can be used as indicator plants in this approach [37]. Such testing is faster as compared with the use of woody indicators. The symptoms develop within 7–10 days [33].

On the other hand, if a virus cannot be transmitted by using the sap of a sick plant, then bud grafting is used. The dormant buds are inoculated to the woody shoots of an indicator plant (sensitive grape variety or species). For example, the indicators used to detect the viruses of the *Closteroviridae*, *Betaflexiviridae*, and *Tymoviridae* families are St. George (*V. rupestris*), LN33 (1613 Couderc × *V. vinifera* cv. Thompson seedless), Kober 5BB (*V. berlandieri* × *V. riparia*) and *V. vinifera* cv. Cabernet Franc [28,38]. However, the duration of such testing in the case of grapevine viruses may be very prolonged. Thus, Rowhani et al. [39] indicated that an assay using a panel of grapevine indicator hosts requires up to 3 years to complete. This specific feature of bio-testing for grapevine viral infection is an essential limitation for the use of grapevine hosts as indicator plants.

With the development of faster and more accurate diagnostic techniques, the use of biological testing has decreased. However, they are applied when new viruses with unknown nucleotide sequences and unproduced antibodies are studied [33,37,40].

4. Immuno-Diagnosis

Immunoassays are based on the binding of an antigen of interest (in our case—viral particle) to specific antibodies. Therefore, preliminary work on the acquisition and description of the antibodies used in the test is needed to carry out such assays. Both poly- and monoclonal antibodies are used in the immune detection of pathogenic viruses. Polyclonal antibodies represent a pool of immunoglobulins that is acquired through the immunization of animals (rabbits, sheep, goats, etc.) with a purified or partially purified preparation of a virus, or a recombinant protein that presents a specific antigenicity of a virus. The technique of polyclonal antibody production is rather simple, but the antibody characteristics vary from animal to animal, so that the ratio of the antibodies to the various antigen determinants is inconsistent, and in some instances, there is also cross reactivity with related viruses [41]. Such problems do not exist when monoclonal antibodies are used. The monoclonal antibodies bind to only certain antigen structures, and they can be produced in unlimited quantities, with no changes in their affinities and specificities.

When purified grapevine virus preparations are used as immunogens, they often result in low concentrations, and an inadequate affinity of produced antibodies [42]. This is why the possibility of antibody production against grapevine viruses through the use of highly conserved recombinant proteins of the viral envelope as immunogens is of great interest. The approach was successfully used by Orecchia et al. [43] to produce antibodies against GLRaV-3, by Saldarelli et al. [44] against GVB, and by Shibaei et al. [45] against GFLV. Studies of Fajardo et al. [46] (immunoassay of *Grapevine leafroll-associated virus 3*) and Koolivand et al. [47] (immunoassay of *Grapevine fanleaf virus*) show the successful use of the non-structural (movement) proteins of viruses as immunogens to garner specific antibodies for analytical purposes.

Recombinant techniques make possible the further production of antibody fragments that preserve antigen-binding properties. For example, Nölke et al. [48] obtained a single chain fragment of the scFv GFLVcp-55 monoclonal antibody specific to the *Arabis mosaic virus* and GFLV. This fragment was produced in *Nicotiana benthamiana* transgenic plants that accumulated scFv in their cytosol (up to 0.1% of their total soluble protein). Orecchia et al. [43] produced a recombinant Fab-fragment of an antibody against the viral envelope protein, named GLRaV-3, with high affinity to a full-sized virus particle.

Immunoassays have less demanding testing conditions than molecular genetic techniques. Sample preparation for virus detection in plant material usually includes the only stage of its grinding in a special buffer. Unlike nucleic acid detection, the immunoassay does not rely on a number of amplification enzymes and is therefore less sensitive to contamination. The enzyme-linked immunosorbent assay (ELISA) results are not influenced by the polysaccharides and polyphenols that are present in plant sap, and these often interfere with the results of the polymerase chain reaction (PCR).

For end-users to be able to carry out an immunoassay, the antibodies produced should be included in commercially-available analytical systems. The development and efficiency assessment of such proposed prototype solutions for the detection of grapevine viruses was used for various kinds of immunoassays, such as ELISA [49], lateral flow immunoassay (LFIA) [50,51], immune-filtration with magnetic nanoparticles [52], immunosorbent electron microscopy (ISEM) [53,54], and direct immune-printing (DIP) [17,55]. The Western blotting method is also of great interest [56–60], because it can possibly not only establish that antibodies bind with sample components, but can also conclude about the presence of certain antigens in the sample, thus contributing to a more specific pathogen characteristic. By this way, the viruses that have not unique antigenic compounds but differ one from the other by their presence or absence cannot be distinguished by ELISA of LFIA (where the total antigen-antibody binding is registered), but will have different set of bands in Western blotting.

The assortment of commercially available immune analytical systems for the detection of grapevine viruses today is mostly represented by ELISA kits.

ELISA is based on a combination of several methodological solutions: the sequential formation of immune complexes in the wells of microtitration plates, the separation of non-interacted reactants

and matrix compounds, and the use of enzyme label to register the formed immune complexes [61]. The widespread use of ELISA is driven to a great extent by the development of technology for the production of a complete set of materials and equipment that are needed for reproducible and high-performance analysis; i.e., microplates with a controlled sorptive capacity, pipettes designed for work with microplates, automated microplate washers, and reasonably priced photometric detectors for measuring the optical density in microplate wells. A standard 96-well microtitration plate enables the simultaneous testing of up to 40 samples in two replications. In the case of plant virus control, the quantitative information about their content is not requested, and positive or negative ELISA results may be estimated visually as the presence or absence of coloration in the corresponding microplate wells. With respect to pathogen detection in plant material, there are quick and simple procedures for sample preparation, consisting of sample grinding and trituration in a specially matched buffer for not more than 10 min.

Usually, carrying out an ELISA takes anywhere from 2–3 h up to 24 h, due to the need to reach chemical equilibrium for all successively conducted analytical reactions. Duration of the stages may be reduced, but such changes leads to non-equilibrium regime of immune interactions with lower quantity of formed immune complexes, increased limit of detection, and/or lower accuracy of the quantitative measurements.

Since grapevine viruses are polyvalent antigens, a sandwich ELISA is used to maximize the detection sensitivity [21,47,62,63]. The principle of this format is explained below, in its double antibody sandwich ELISA (DAS-ELISA) version. The antibodies against the virus of interest (first antibodies) are adsorbed onto the microplate surface. In a commercial test system, this is already done by the manufacturer, and users receive plates with the antibodies already immobilized. The prepared samples (plant extracts that may contain viruses) are added to the microplate wells. During the incubation, the first antibodies—the antigen complexes are formed. After washing, second virus-specific antibodies are added, which are covalently labeled by an enzyme. Unlike many other ELISA application areas, in plant sample analyses the alkaline phosphatase is the predominantly used label. The reason for this is that the activity of a more conventional peroxidase is sensitive to the bio-sample matrix components, despite the fact that they are added to the wells at different stages. During the next incubation, the first antibody–antigen–the second labeled antibody complexes are formed, whereas unbound compounds were removed from the wells. These complexes are detected by adding the enzyme substrate.

Poly- and monoclonal antibodies, their mixtures, and polyclonal antisera are used as the first and second antibodies in ELISA. There are also modifications of ELISA protocols with more sophisticated assemblies of immune complexes. For example, Boscia et al. [64] immobilized the polyclonal antibodies against the GVA through staphylococcal protein A onto the surface of a polystyrene plate, and the monoclonal antibodies were conjugated with alkaline phosphatase. The use of protein A makes the oriented immobilization of the first antibodies possible, and consequently, this leads to a greater number of available reactive groups on the surface of the microplate.

Alongside DAS-ELISA, the Triple Antibody Sandwich ELISA (TAS-ELISA) is also used, in which the anti-viral labeled antibodies are replaced with successively added non-modified anti-viral antibodies and enzyme-labeled anti-species antibodies. For example, Abdullahi and Rott [21] conducted a TAS-ELISA of grapevine viruses.

In an ELISA of the grapevine viruses, the green parts of the plant (leaf blades, leaf petioles) and the phloem scrapings from the woody part of a vine are usually tested. The virus may be unevenly distributed in a plant, and the virus contents may vary significantly depending on the season. This is why, when it comes to various pathogenic viruses, there are additional recommendations regarding the sampling time and the biomaterial types preferred for testing. A reasonable recommendation is for several leaves to be sampled from a plant, together with their stalks, and then combined in a mixed sample for testing. The collected samples are ground and triturated with an extraction buffer containing additives, such as polyethylene glycol, polyvinyl pyrrolidone, detergents, stabilizers, etc. [49,62]. A typical sample buffer ratio is 1:10. The composition of extraction buffers in commercial

test systems is not disclosed. After the extraction, the solid particles are separated by centrifugation or filtration.

A specific feature of grapevine virus ELISA is only qualitative assessments of the obtained results. The tested samples are compared with positive and negative standard preparations from the ELISA kit, and conclusions on the presence or absence of pathogenic virus in the samples are made. Besides, ELISA is used for research purposes as a quantitative assay to characterize content of virus in different parts of a plant or to study changed content of virus in the course of disease. For example, the range of detectable virus concentrations for the GFLV ELISA developed by Rettcher et al. [52] ranged from 6 ng/mL to 20 μg/mL. The ELISA for the GLRaV-3 developed by Cogotzi et al. [49] was calibrated by using the preparation of an envelope recombinant protein, and it was established that its detection limit was equal to 0.1 ng per well.

Currently, a number of ELISA kits allowing for the detection of different grapevine viral pathogens are commercially available. The key information on these systems is provided in Table S1 (see Supplementary Materials).

Abdullahi and Rott [21] described an interesting combination of ELISA with a microarray technique. They used contact printing to immobilize specific antibodies onto a modified glass sheet, forming binding zones with immune reagents of different specificities. Further analysis was conducted with double or triple sandwich ELISA with chromogenic or fluorogenic substrates. This microarray ELISA had a sensitivity, specificity, and duration that were similar to that of conventional ELISA, with the same antibodies. Although microarray immunoassays in a variety of described versions require more sophisticated equipment in comparison to the conventional microplate ELISA, these approaches are of apparent interest in the case where simultaneous checking of samples for several pathogens is necessary.

The second main direction in immunodiagnosis is based on lateral flow immunoassays (LFIAs).

The main requirements to screening analytical techniques are the reduction of their duration and labor intensity. Among different immunotechniques, the LFIA satisfies these requirements the most. This analysis is based on the use of a multi-membrane composite (test strips), the components of which are pre-treated with all the reagents necessary for the formation of selective immune complexes and their visualization. The contact between the test strips and the sample makes the liquid move along the test strip under the influence of capillary forces. During this movement, specific immune interactions with the pathogenic virus that is potentially present in the sample take place. After the completion of the liquid movement (which usually takes 5–15 min), some zones of the test strip change color due to the binding of the immune reagents and the stained label. The presence of pathogens in the sample is assessed visually (a coloration appears in the zone of specific binding). LFIA also may be used to quantify the content of pathogens. The intensity of coloration in the binding zones is registered for this purpose and compared with values from a calibration curve. The existing devices (including commercially available ones) and software for such registration in LFIA of different compounds are considered in [65–67].

When it is used for phytopathogen detection, LFIA shows a number of advantages: (1) efficient diagnosis in unequipped laboratories and in non-laboratory conditions; (2) quick analysis with minimal sample preparation; (3) one-stage analysis not requiring additional reactants or manipulations; and (4) simple detection and interpretation of the results [68–71].

Although LFIA was developed and used by many investigators for the purpose of viral and bacterial phytopathogen diagnosis [72,73], the literature provides no data on the development of the LFIA test systems for grapevine virus diagnosis. There are only two works that have been published on the detection of the GLRaV-3 and GFLV viruses [50,51] by using commercial test stripes that are not present in manufacturers' catalogues thus far.

As of today, only two LFIA test systems are mass-produced, i.e., the system for *Arabis mosaic virus* detection (Agdia, Elkhart, IN, USA) and for GLRaV-3 virus detection with magnetic concentration requiring additional instrumentation (Bioreba, Reinach, Switzerland).

Immunochromatography (lateral flow) closely resembles immune filtration (through flow) analysis. Although the latter is more difficult to carry out in comparison to the lateral flow, because it involves antibody-mediated analyte binding on a membrane during the filtration of large sample amounts, and the subsequent detection of the resulting complex using enzyme-labelled antibodies, the through flow technique has certain advantages. It can concentrate the analyte from a large volume of the filtered sample onto a small membrane surface, thus lowering its limit of detection. Rettcher et al. [52] developed an analysis that included immune filtration with subsequent detection, and a quantitative assessment of viruses labelled with magnetic beads. The authors immobilized the monoclonal antibodies against the GFLV capsid protein on through flow columns. The same antibodies were bound with the magnetic nanoparticles and used to obtain sandwich immune complexes (antibody—virus—nanoparticle-labelled antibody). The magnetic beads are used in this assay as detected labels, the viral titre was determined by measuring the magnetic signal of the bound nanoparticles. The analysis time was 30 min.

5. Diagnostic Methods Based on Nucleic Acids Amplification

With developing technology, the labour-intensive and time-consuming biological methods of diagnosis are replaced by fast and accurate methods of nucleic acid or viral protein molecular identification. One such technique is the PCR, which is based on the repeated copying of viral genome fragments by the *Taq* polymerase enzyme. Presently, various PCR modifications are used for different purposes: PCR with reverse transcription (RT-PCR), real time PCR (qRT-PCR), multiplex PCR, nested PCR, and so on.

PCR is used for the detection of grapevine DNA viruses, for example, the GRBaV [15,74]. To detect the RNA viruses in a plant, PCR is carried out on the complementary DNA (cDNA) matrix that results from a reverse transcription reaction on the extracted plant RNA [75–78]. PCR and RT-PCR are often used in studies of viral genetic diversity [51,79–82]. RT-PCR itself is used to confirm the results of serodiagnosis [83,84].

A shortcoming of conventional endpoint PCR is that it necessitates additional visualization of the products in an agarose gel, which increases the analysis time and the contamination risk. Moreover, false-negative results are possible in grapevine virus diagnosis, due to PCR inhibition by phenolic compounds and polysaccharides from grapevine tissues [85]. These problems are partially solved by using qRT-PCR that is based on a measurement of the synthesized product amount by using fluorophores or intercalating dyes [86].

The protocols involving the fluorescent TaqMan® probes are designed to detect the most important grapevine viral pathogens: GLRaV-1–5, -7, and -9 [74,86–88], GVA, GVB, GVD [89], GFLV [86,90], GFkV [86], and GPGV [91,92]. The intercalating dyes (SYBR green etc.), are also widely used for grapevine virus diagnosis [93–96]. The conducted studies show that the qRT-PCR sensitivity is higher than that of the ELISA [90,97], as well as that of the conventional PCR and RT-PCR methods [74,86,87].

An apparent advantage of the PCR is the possibility to detect several pathogens in one reaction [85,98,99]. This involves the matching several primer pairs to amplify the genomes of the various viruses, thus reducing the cost of analysis and the time required. The growing popularity of this PCR modification is limited by the necessity to select common conditions for efficient amplification of all primers. The sensitivity of multiplex qRT-PCR is on a par with classical PCR, and significantly higher than the sensitivity of the ELISA [99–101].

qRT-PCR using TaqMan® probes is the most popular approach for the multiplex detection of grapevine viruses. Lopez-Fabuel et al. [100] described a technique for the simultaneous identification of five viruses: GFLV, ArMV, GLRaV-1, GLRaV-3, and GFkV. A quadruplex detection protocol was developed for nine economically significant grapevine viruses [102].

Nested PCR was developed to increase the assay sensitivity. Here, an amplification product of the first reaction was used as a matrix during the second PCR with another primer pair, thus contributing to its increased specificity and sensitivity [99]. Nested PCR underlies several protocols for the detection

of Nepovirus genus viruses, and these techniques make the diagnosis possible, with the sensitivity being several times higher than that of RT-PCR [103]. This method was used for studying the genetic diversity of GLRaV-1, GLRaV-3, and GFLV [77,104,105].

One more modification of PCR is droplet digital PCR, which is being increasingly and more widely used in recent phytopathogen diagnoses [106–108]. This method relies on dividing the PCR mix into small droplets, with amplification and signal identification taking place in each of the droplets. EvaGreen intercalating dye or fluorescent probes can be used for this purpose. In comparison to qRT-PCR, such an approach provides increased sensitivity, less affected by inhibitors, and the ability to detect the absolute quantity of pathogens without building a calibration curve [108–110]. This method was successfully used for GRBaV detection [111].

To simplify PCR, a loop-mediated isothermal amplification (LAMP) method was proposed, which involves the amplification of specific DNA sequences using four or six primers [112]. The advantages of LAMP are fixed temperature conditions, high specificity, increased speed, visual detection, and resistance to inhibitors [113,114]. The sensitivity of this reaction is either higher than, or similar to that of PCR [107,114]. LAMP was used for the detection of grapevine DNA phytopathogens [107,115–118]. Combined with reverse transcription in one tube, LAMP was used for GLRaV-3 detection [113]. Due to its high sensitivity, this method makes it possible to detect one positive sample in a composite sample of 50 specimens. A comparison of the various methods shows that RT-LAMP is as sensitive as that of nested PCR, and that it presents a possible alternative to ELISA for the quick detection of GLRaV-3 with minimal equipment [113]. During GFLV detection, RT-LAMP shows a higher sensitivity than DAS-ELISA, RT-PCR, or immuno-RT-PCR, while immuno-RT-LAMP shows the highest sensitivity in comparison to all of the above-named techniques [114].

Methods based on the amplification of virus nucleic acids are one of the most common for current routine diagnostics. The variety of commercially available tests is presented in the Supplement (Table S2). The sensitivity and specificity, relatively low cost, comparatively short analysis time, as well as the possibility of using some amplification modifications under field conditions, are all factors that increasingly broaden PCR-diagnosis application for grapevine disease control.

6. DNA Microarrays

One more diagnostic method based on the detection of viral nucleic acids is the DNA microarray. A DNA microarray is a substrate to which probes for the simultaneous detection of several targets are covalently attached. During DNA microarray analysis, nucleic acids are hybridized with a probe. The data on the hybridized probes are used to identify the sequences that are present in the sample. Using these microarrays in the diagnosis of plant diseases, tens and even thousands of pathogens can be identified simultaneously. In experiments on virus detection in plants, microarrays usually show higher sensitivities than ELISA, but lower than qRT-PCR [119]. For routine diagnosis of a certain pathogen, the use of the DNA microarrays is less costly than next generation sequencing (see Section 7).

The first time that the technique of low-density DNA microarrays was used in the detection of grapevine viruses, it found 13 viruses [89]. The mixtures of primers and TaqMan® probes were dried onto the surface of a plastic 384-well plate, after which the cDNA was added, and the qRT-PCR was conducted. Thus, implemented DNA microarray techniques show maximum sensitivity and reproducibility, in comparison to a single-stage RT-PCR and a single-stage qRT-PCR with TaqMan® probes. Later, a 70-dimensional oligonucleotide microarray was developed for the simultaneous detection of a large number of grapevine viruses [120], and 570 unique probes that were complementary to the sequences of 44 plant-virus genomes were attached to the substrate. Subsequently, the use of this approach to certify the material, as well as to identify new viruses in which the genome sequences are partially complementary to the probes, is suggested.

A possible use of a DNA array without additional amplification was demonstrated for the detection of 15 grapevine viruses: eight nepoviruses, two vitiviruses, and one virus for each of the Ampelovirus, Closterovirus, Maculavirus, Foveavirus and Sadwavirus genera [121]. Furthermore,

a microarray technology with preliminary cDNA amplification was applied for 33 samples, for the simultaneous identification of five grape leafroll viruses [122]. The authors also demonstrated the possibility of identifying a mixed viral infection. In a modified chip version [123], where 1578 virus-specific and 19 internal probes were used, of 60–70 nucleotides in size, it is possible to detect 38 viruses from different families. The viruses were identified both in single virus and mixed infections, and the results were confirmed by both RT-PCR and ELISA. Consequently, it can be concluded that a DNA microarray could be used to describe the viruses, certify the material, and provide a quick diagnosis of the material being imported.

The possibility of the simultaneous detection of a large number of viruses by one assay using DNA chips is essential for the diagnosis of grapevine infections. Since mixed infections with several pathogens at a time is an often-seen constellation in grapevine plants [124], this feature of the DNA chip technique is a significant advantage not only for research purposes, but for further commercialization. Actually, commercial DNA chips are available only for other plant diseases (for example, foe pathogens of potato for «Bioreba»), but not for grapevine viral pathogens.

7. Next-Generation Sequencing: A New Approach to Viral Diagnosis

Next-generation sequencing (NGS) propels phytopathogen detection to a new level. Techniques based on PCR and immunotechniques are applicable only if there is information on the genome of the virus of interest, or available antibodies specific to the viral proteins. Therefore, new disease-causing viruses cannot be detected with this method. The resulting information indicates not only the presence of a specific virus, but also its genomic structure and integrated information on all of the viruses that are present in the sample [28,124–130].

There are several ways of using NGS for virus detection. One of the techniques was implemented by Adams et al. [125], and involved total RNA sequencing and subtractive hybridization, in order to decrease the amount of non-viral RNA reads. When this approach is used, however, viruses with a low titre may not be detected, due to losses encountered during nucleic acid extraction. Another approach implies virus detection based on the sequencing of the small RNAs that are formed in a plant as a result of RNA silencing in response to a viral infection or as part of a stage in the viral life cycle [126,130]. In this case, it is possible to detect not only familiar viruses with a very low titre in a plant, but also plant viruses that have not been described. Another approach is to sequence dsRNAs from viruses, which leads to an increase in virus-related reads [126,127]. Possible limitations of this approach are associated with a small amount of replicative intermediate RNAs [131].

NGS was first used for grapevine plants when studying the aetiology of the Syrah variety of plants [126]. By total plant RNA sequences using Life Sciences 454, not only familiar viruses (GRSPaV; *Grapevine rupestris vein feathering-associated virus*, GRVFV; GLRaV-4; GLRaV-9) and viroids (*Australian grapevine viroid*, AGVd; *Grapevine yellow speckle viroid*, GYSVd; *Hop stunt viroid*, HSVd) found, but a new virus (*Grapevine Syrah virus 1*, GSyV-1) was discovered as well. The potential of NGS was also shown in the study of the vineyard virome by the sequencing of double-stranded RNAs (dsRNAs) extracted from 44 random plants [127]. Already-known viruses—GLRaV-3, GRSPaV, GVA, and GVE—were detected. NGS can be further used to study genetic biodiversity and the spread of viruses and viroids [78,128,132–138].

Therefore, an undeniable advantage of the NGS technique is that it makes the detection of new viruses possible. For example, during an analysis of the small RNAs of a grapevine showing symptoms of unknown aetiology, the Grapevine vein-clearing virus (GVCV) with a double-stranded DNA genome [15] was found. Al Rwahnih et al. [139] discovered a new Grapevine virus F (GVF) Vitivrus in Cabernet Sauvignon plants by dsRNA sequencing using the Illumina platform. Via an analysis of small RNA, Giampetruzzi et al. [140] describe GPGV Trichovirus in vines with symptoms of chlorotic spots and leaf distortion. Rwahnih et al. [16] were the first to detect the GRBaV virus of the *Geminiviridae* family. In this study cDNA libraries from dsRNA were used for sequencing on an Illumina platform. NGS of siRNAs was used by Maliogka et al. [141] to initially detect Grapevine

Roditis leaf discoloration-associated virus (GRLDaV) Badnavirus. The list of the new grapevine viruses detected using the NGS technique is quickly growing, due to recent studies [8–11,134]. However, this is only the first step in the study of new viral diseases, which should be followed by an assessment of the contribution of the virus to the development of symptoms, the study of its biology and ecology, the need to correct regulatory documents and certification schemes. For these purposes, biological indexing methods are indispensable.

High costs limit the application of NGS in routine diagnosis. Additionally, the wider use of this technique is limited, due to its requirement for processing sequencing data using bioinformatics methods [142]. To obtain the most complete and reliable information on the presence of viruses, it is necessary to develop a single pipeline for processing bioinformatic data. In this regard, bioinformatics processing methods are discussed and improved [143,144]. Their harmonization will enable NGS to be included in certification schemes.

The technology behind NGS is developing rapidly. New solutions appear frequently, such as the sequencing of RNA or DNA single molecules without amplification. In light of these trends, it is very likely that NGS will become more frequently used in the future for the certification of planting material, checking stools, the detection of new viruses, and the aetiological identification of previously unknown diseases [129,145].

Studies involving NGS show that basically every grapevine plant contains viruses or viroids. Some viruses persist in the plant almost permanently without causing any symptoms. This is why the concept of a 'plant free from viral infection' is, in most cases, not applicable to a grape plant [124]. It is only possible to conclude that the plant has no pathogens of economic importance. Thus, the development of diagnostic techniques for grapevine viruses calls for a review of the planting material assessment criteria. The requirement of virus-free plant would be transformed in the future to the requirement of the absence of "economically most dangerous" pathogens.

8. Conclusions

The possibilities and limitations of the existing techniques for the diagnosis of viral grapevine pathogens can be summarized by Table 2 below. We can conclude that visual diagnosis is a starting point for further activities in confirmation of conclusions and strong identification of pathogenic virus. PCR diagnostics and immunoassays are efficient confirming assays for known priority viruses. Their use allows assess the spread of a detected virus. The choice between PCR diagnostics and immunoassays depends on available equipment and reactants. Row of commercial immunoanalytical kits may be insufficient for strong identification of specific virus. However, when monitoring the known priority pathogens, the immunochemical control will be more productive, and in the variant of immunostrips - more rapid. NGS techniques provide virus detection and biological indexing. This information from specialized laboratories indicates pathogens for further investigating and possible monitoring. NGS data are also used to design PCR diagnostics and to choose potential antigens for specific immunodetection of novel viruses. Thus, the currently known advanced laboratory methods used in research, integrated with biological indexing, allow us to identify new pathogens and develop methods for their routine diagnostics both in the laboratory and in the field.

Table 2. Summary of the methods used for grapevine virus detection.

Method	Requirements for Personnel Qualification	Requirements for Equipment and Data Processing	Time-Consuming	Field of Application
Visual diagnosis	Significant practical training is required	No special requirements	Several minutes	Field diagnostics
Biological testing	Significant practical training is required	Collection of indicator plants, equipment for year-round use	May take from several weeks to 2–3 years	Research, certification
ELISA	Moderate	Specialized serial equipment with a relatively low price	Several hours	Research, certification, routine laboratory diagnosis
LFIA	Low	Simple equipment, possibility of visual results assessment	10–20 min	Field diagnostics
Nucleic acid amplification	Moderate	Special equipment, the price for the equipment can be rather high	From dozens of minutes to dozens of hours	Research, certification, routine laboratory diagnosis, field diagnostics (isothermal amplification)
DNA microarrays	High	Special equipment	Dozens of hours	Research
Next generation sequencing	High	Special high-cost equipment, specialized data processing	Dozens of hours	Research

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/8/12/195/s1, Table S1: The main ELISA test systems for the control of grapevine virus pathogens that are commercially available, Table S2: The main commercial kits based on nucleic acid amplification for grapevine viruses diagnostic.

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