Diagnosis of Plant Virus Diseases

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Among the plant pathogens, viruses (and viroids), which are transmitted by a living organism called a vector, are the major infectious agents that cause plant disease. Once a plant virus infects a susceptible host, it can spread to another plant by means of vector-mediated transmission (horizontally) or from parents to offspring (vertically). Since accurate diagnosis methods are of pivotal importance for viral diseases control, the current and emerging technologies for the detection of these plant pathogens are described.

fruit disease plants virus

1. Introduction

Trees and herbs that produce fruits represent the most valuable agricultural food commodities in the world. However, the yield of these crops is not fully achieved due to biotic factors such as bacteria, fungi, and viruses. Viruses are capable of causing alterations in plant growth and development, thereby impacting the yield of their hosts significantly.

2. Diagnosis of Plant Virus Diseases

Viruses can cause huge economic losses by affecting the quality and productivity of various fruit plant crops like banana, apple, grapevine, citrus, and others [1]. Once infected, plants harbor the virus throughout their life, and visible symptoms of viral diseases appear. So far, it is very difficult to prevent the spread of viruses and their vectors into new territories through international trade, mainly given by the exchange of plant materials across borders [2]. Therefore, early diagnosis of viral diseases is a key factor that determines the timely use of protective measures to confine the viruses, thereby preventing yield losses and a decrease in the quality of fruit products.

The first way to detect viral infections was according to the symptoms they produced. Such an approach was based on biological indexing tests, which were time-consuming and unreliable, especially in cases in which the virus infection was latent or plants exhibited virus-like symptoms unrelated to virus etiology [2][3]. Then, virus detection at the microscopic level began in the late 1930s with electron microscopy; however, this technique cannot be applied for large-scale detection due to the dimensions and costs of the equipment, as well as the operating conditions. Subsequently, the application of serological assay like enzyme-linked immunosorbent assay (ELISA) [4] and nucleic acid-based assay for in vitro DNA amplification called polymerase chain reaction (PCR) [5] represented important advances in plant viral diagnosis. With the progress in molecular biology, nucleic acid-based techniques evolved significantly from conventional PCR to reverse transcription PCR (RT-PCR), nested PCR, multiplex PCR,

real-time PCR, immunocapture-PCR (IC-PCR), loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), and microarray and next-generation sequencing (NGS) to improve specificity, sensitivity, and multi-sample processing [2].

2.1. Serological Assays

2.1.1. ELISA

ELISA is a cost-effective and robust assay for the routine detection of plant viruses, especially those present in crude extracts, due to its simplicity, detection limit 1–10 ng/mL, ability to analyze a large number of samples, easy interpretation, and semi-quantitative results [6][7]. For virus diagnosis, it is based on the detection of viral antigens, mainly the components of viral particles such as coat protein (CP) subunits, with enzyme-labeled antibodies. The amount of virus present is proportional to the amount of enzyme-labeled antibody, and it is detected by a colorimetric reaction with the enzyme substrate [4]. The direct and indirect ELISA are the most used methods for the diagnosis of plant viruses [8]. Compared to the direct antigen-coated ELISA (DAC-ELISA), the double antibody sandwich ELISA (DAS-ELISA) is the most used test as it is more virus-specific. Currently, DAS-ELISA-based diagnostic kits are used in accredited testing laboratories for routine virus indexing and certification programs for most horticultural crops, including EFFs [9]. A further improvement in the ELISA introduced the use of monoclonal antibodies (MAbs), which specifically detect a particular virus [9]. However, the main limitation of ELISA is the good quality and quantity of antibodies. For example, mixed infections are common in crops of grapevines and citrus [10] [11], making it impossible to isolate individual viruses and thereby hindering the obtention of specific antibodies from detecting a particular virus [12]. Recombinant proteins, on the other hand, are an alternative approach for immunogen preparation, especially for viruses in low concentrations. The recombinant approach is fast, economical, and overcomes the problems associated with conventional antigen purification [13]. Additionally, the expression plasmids can be stored for long periods of time, and the recombinant viral proteins are uniform, therefore reducing non-specific antibody recognition. These recombinant proteins are used successfully for polyclonal antibodies (PAbs) production against viruses infecting papaya, bananas, and grapes and to develop immunodiagnostics for routine testing [14][15][16]. In this regard, an important advance was made by producing cocktails of PAbs against the recombinant fusion CP of two and three viruses infecting vegetable crops [17]. Dual and triple cocktails of PAbs were generated for DAC-ELISA toward the detection of CMV and papaya ringspot virus (PRSV; *Potyvirus*, *Potyviridae*) or CMV, PRSV, and groundnut bud necrosis virus (GBNV; *Orthotospovirus*, Tospoviridae) simultaneously in *Cucurbitaceae*, *Solanaceae*, and other hosts [17].

2.1.2. Lateral Flow Assay (LFA)

LFA is an ELISA variant that simplified virus identification [18]. LFA can be done anywhere, with a simple device operated by personnel with little or no training, and results are obtained within minutes [19]. This membrane-based assay involves the use of specific MAbs and PAbs in an immunochromatographic format, incorporating antibody-coated gold or latex particles. A lateral-flow strip consists of a sample application pad, a conjugate or reagent pad, a reaction membrane, and an absorbent pad [20]. There are two types of LFA, the double antibody sandwich format and the competitive format [21]. The sandwich format contains a virus-specific antibody that is immobilized on a

membrane, as the test line that captures the viral protein, and a detection antibody-specific antibody is deposited in the control line, capturing the unbound conjugated antibodies. The appearance of the test line indicates the presence or absence of the virus in the sample, and the control line is an internal control to ensure proper functionality of the test [20]. In the competitive format, on the other hand, the detection signal correlates negatively to the antigen concentration. Additionally, these tests have been combined with a novel extraction procedure to allow disease diagnosis in the field. The LFA was successfully applied for the onsite rapid detection of CTV infecting citrus, satsuma dwarf virus (SDV; *Sadwavirus*, *Secoviridae*), and plum pox virus (PPV; *Potyvirus*, *Potyviridae*) infecting apricot, plum, and peach [19][22][23]. LFA has also been useful for the diagnosis of CMV infecting pepper, cucumber, and melon crops, tomato spotted wilt virus (TSWV; *Orthotospovirus*, *Tospoviridae*) infecting pepper and tomato plants [24][25], and PepMV infecting tomato and sweet cucumber [26]. Finally, the development of lateral flow strips has allowed the onsite detection of TBRFV, squash mosaic virus (SqMV; *Comovirus*, *Comoviridae*), tobacco etch virus (TEV; *Potyvirus*, *Potyviridae*), TMV, tobacco ringspot virus (TRSV; *Nepovirus*, *Secoviridae*), tobacco streak virus (TSV; *Ilarvirus*, *Bromoviridae*), ToLCNDV, ToRSV, ZYMV, CGMMV, melon necrotic spot virus (MNSV; *Gammacarmovirus*, *Tombusviridae*), melon severe mosaic virus (MSMV; *Orthotospovirus*, *Tospoviridae*), PepMV, pepper mild mottle virus (PMMoV; *Tobamovirus*, *Virgaviridae*), potato virus X (PVX; *Potexvirus*, *Alphaflexiviridae*), and PVY, among others [27].

2.1.3. Dot Immunobinding Assays

Serological assays such as dot immunobinding assay (DIBA) and tissue blot immunoassay (TBIA) also allow simultaneous screening of large numbers of samples [28]. In these assays, the sap from infected plants is blotted on nitrocellulose or nylon membrane, and the virus is detected by enzyme-labeled secondary antibodies or chemiluminescent probes. DIBA is considered to be simple, rapid, and often more sensitive than ELISA-based techniques [29]. DIBA was adapted for detection of CTV and compared with DAS-ELISA and DAS-indirect ELISA; this assay was easier to perform and as sensitive as either ELISA procedure for CTV diagnosis [30]. This technique was also able to detect the infection with ACLSV and ASGV [31]. Moreover, TBIA was useful for the detection of CMV [32][33].

2.1.4. Computer-Assisted Epitope Identification to Improve Antibody Production

New approaches are emerging in order to improve serological diagnosis. Some involve the identification of epitopes in the viral CP by computer simulation or functionally by epitope mapping and its subsequent artificial synthesis [15]. According to the second approach, two putative CP coding regions (p48 and p37) of banana streak MY virus (BSMYV; *Badnavirus, Caulimoviridae*) were identified in silico by comparison with caulimoviruses, retroviruses, and rice tungro bacilliform virus. A purified fusion protein p37 was injected in rabbits as an antigen for raising polyclonal antiserum. The antiserum was successfully used in antigen-coated plate-ELISA for specific detection of BSMYV in banana fields and tissue cultures raised. On the other hand, the antiserum was also utilized in immuno-capture PCR (IC-PCR) for indexing of episomal BSMYV infection [15]. This bioinformatic approach can be useful for the precise location of CP coding sequences that are not available in any virus.

2.2. Nucleic Acid-Based Assays

2.2.1. PCR

Molecular diagnostics began in the mid-1980s with the introduction of PCR, and the first PCR method for virus detection was published ten years later [34]. PCR-based diagnostics can be used for the detection of viruses with DNA and RNA genomes. In the case of RNA viruses, RNA is first reverse transcribed into complementary DNA (cDNA) in a process known as reverse transcription (RT), followed by conventional PCR, involving the amplification of target nucleic acid sequences with primers [35]. Primers can be easily designed using the viral sequence information in databases. Another crucial component for successful PCR is the isolation of good quality DNA, free of endogenous polyphenols, polysaccharides, and nucleases. Some crops, such as bananas, contain very high levels of polyphenols and other secondary metabolites that interfere with PCR amplification. Therefore, an efficient method adapted for nucleic acid isolation is important [2].

Many commercial nucleic acid extraction kits (e.g., RNeasy and DNeasy) have replaced complicated and time-consuming conventional nucleic acid extraction protocols [36][37][38]. PCR-based methods allow the precise detection and characterization of plant viruses, as the amplified products can be sequenced directly or cloned into a suitable vector. Besides reverse transcription PCR, there are other diagnostic techniques based on PCR [39]. *Nested PCR* is useful when the virus titer is very low, the target gene is unstable, and it cannot be checked by electrophoresis due to low amplification product. The product from primary PCR amplification is used for second PCR amplification. Several viruses, including prunus necrotic ringspot virus (PNRSV; *Ilarvirus*, *Bromoviridae*), prune dwarf virus (PDV; *Ilarvirus*, *Bromoviridae*), PPV, and CTV, were detected by this technique. *Co-operational PCR* also requires four primers; however, one is external, and three are internal primers instead of two external and two internal primers associated with nested PCR. This technique shows similar sensitivity to nested PCR, detection in real-time, capability of coupling with dot blot hybridization, it can avoid false-positive results shown at nested PCR, and it also can be applied to capillary air thermal cyclers. This technique has been useful to detect cherry leaf roll virus (CLRV; *Nepovirus*, *Secoviridae*) with higher sensitivity than RT-PCR. *Digital PCR* does not require any reference standards for nucleic acid quantification but rather produces an accurate quantification. The PCR sample is divided into thousands of nanodrops, and after amplification, the drops containing the sequence of the target DNA are detected by fluorescence as positive and those without fluorescence as negative. Then, the statistical analysis of the number of nanodrops gives an exact count of the target DNA, and this helps count the viral charge. Reverse transcriptase droplet digital PCR detected PMMoV in the presence of qPCR inhibitors [40]. Finally, *Multiplex PCR*, *Real-time PCR*, and *Immunocapture-PCR* are described in the following sections. Importantly, PCR has been used successfully for the detection of several viruses that infect citrus in India, including CTV, citrus yellow mosaic virus (CYMV; *Badnavirus*, *Caulimoviridae*), and Indian citrus ringspot virus (ICRSV; *Mandarivirus, Alphaflexiviridae*) [41]. PCR-based diagnostics are also available for viruses that infect bananas, such as BBTV and BSV [42][43] as well as for GLRaV-3 infecting grapevines [44].

2.2.2. Multiplex PCR

Multiplex PCR is a technique for simultaneous and sensitive detection of different DNA fragments in one single PCR reaction [45], and it allows to save reagents and time. Most of the fruit plant crops are infected by more than

one virus (**Figure 1**) containing DNA or RNA genomes that can be detected by multiplex PCR [46][47][48]. Multiple detection is achieved by combining multiple pairs of oligonucleotide primers, each designed to amplify the desired target. Multiplexing requires to design primers that do not show self-complementarity and that exhibit very similar annealing temperatures. Then, PCR products are distinguished by their size or fluorescent tag [48][49][50]. For the purpose of detecting pathogenic microorganisms, multiplex PCR can be performed in various modalities [48]. *Reverse transcription-multiplex PCR* can simultaneously detect various target RNA. After reverse transcription of target RNA, cDNAs are simultaneously amplified with a set of specific primers in a single tube by multiplex PCR. In order to quantify viral load, this technique can be performed in real-time mode, known as *reverse transcription-real-time multiplex PCR*. *Real-time multiplex PCR* uses a set of species-specific primers and probe that is labeled with different fluorescent dyes for each target species so that approximately 2–5 species (depending on the experimental conditions) can be detected simultaneously in a single real-time PCR reaction. Compared to real-time single PCR, real-time multiplex PCR shortens the processing time and reduces the use of reagents. Multiplex RT-PCR has been successfully applied for aphid-borne viruses infecting strawberry, such as strawberry crinkle virus (SCV; *Cytorhabdovirus*, *Rhabdoviridae*), strawberry mild yellow edge virus (SMYEV; *Potexvirus*, *Alphaflexiviridae*), strawberry mottle virus (SMoV; unassigned genus, *Secoviridae*), and strawberry vein banding virus (SVBV; *Caulimovirus*, *Caulimoviridae*) [51]. Furthermore, this technique has been used for simultaneous detection of several viroids within the family *Pospiviroidae* and the ASGV infecting citrus plants [49]. Another example of simultaneous detection by multiplex RT-PCR is the case of viruses infecting grapevines, apples, bananas, and pome fruits [52][53][54][55], as well as for the combination of several viruses and greening bacterium infecting citrus plants [56]. One of the major limitations of multiplexing is the long time taken for optimization of the annealing temperature of the multiple primer sets and the decrease in sensitivity of detection. Additionally, different targets can compete with each other in the reaction in such a way that targets in very low amounts will be hindered by those in high abundance.

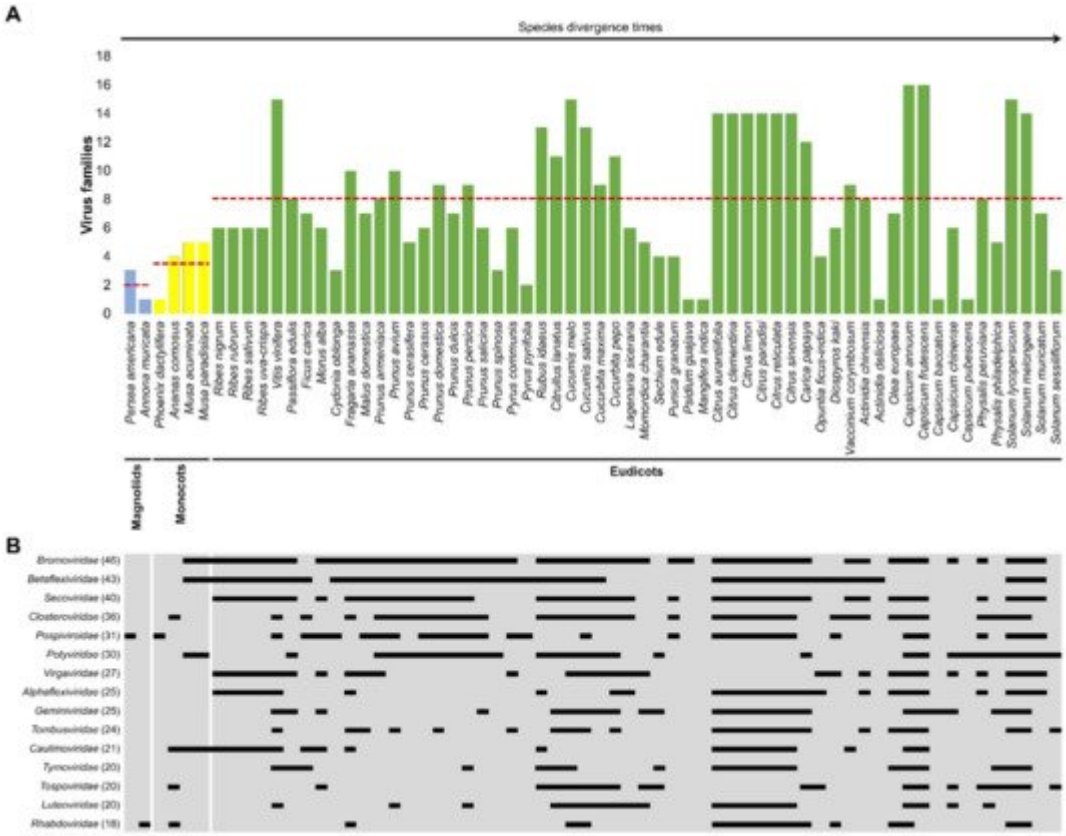

Figure 1. Virus families infecting the 64 commercially important EFFs. (**A**) Classification of EFFs according to plant divergence times [93], and references therein], as well as the number of virus families infecting them. The average number of virus families infecting EFFs classified into magnoliids, monocots, and eudicots is indicated with a red dashed line. (**B**) The host range for the first 15 virus families is exemplified with thin rectangles (filled).

2.2.3. Real-Time PCR

One of the limitations of PCR and RT-PCR for virus detection is that PCR products require agarose gel staining with fluorescent dyes such as ethidium bromide, SYBR Green, SYBR Gold, SYBR Safe, Eva Green, GelRed, EZ-Vision, among others [57], which is not convenient for high throughput applications. Additionally, the amount of PCR product is not proportional to the amount of target DNA, and contamination due to the opening of tubes can lead to false-positive results. The real-time quantitative PCR assay is a tool for the detection and quantification of plant viruses. Real-time PCR eliminates agarose gel electrophoresis usage and allows to determine the increase in the amount of amplified DNA through a fluorescent signal [58][59]. It requires the use of nonspecific fluorescent dyes (e.g., SYBR green, LUX, etc.) or specific probes labeled with fluorescent dyes (e.g., TaqMan, Molecular beacon, etc.). The SYBR green dye binds nonspecifically to dsDNA molecules; therefore, the fluorescence generated could be due to specific or nonspecific amplicons or primer dimers. In such cases, melting profiles are used to discriminate primer dimers from actual amplification [9]. On the other hand, probes labeled with fluorescence are specific because more than two independent oligonucleotides need to bind to the target to generate the signal. In contrast to the development of antibodies required for serological tests, real-time PCR has been successfully used for the specific and sensitive detection of viruses [9]. Even though real-time PCR requires very expensive

equipment, the overall cost for antibody development is much higher. *Reverse transcription-real-time multiplex PCR* allows detection of various target RNA through simultaneous amplification of cDNAs, produced by reverse transcription, with a set of specific primers in a single tube by multiplex PCR, producing quantitative results [39]. On the other hand, *nested real-time reverse transcription PCR* is a simple and sensitive method for the detection of pathogenic microorganisms. This technique involves a previous reverse transcription step to synthesize cDNA and the use of two external and two internal primers that are complementary to the target sequence, which increases sensitivity, providing quantitative results [39]. In summary, the main advantages of real-time PCR assay are the specificity and speed, high throughput testing, detection of low viral titer, and lesser risk of contamination. For instance, real-time RT-PCR has been successfully used for quantitative detection of CTV and citrus yellow vein clearing virus (CYVCV; *Mandarivirus*, *Alphaflexiviridae*) [59][60][61]. Finally, simplex and duplex real-time PCR assays have been developed for the rapid and sensitive detection of GLRaV-3 and grapevine red blotch virus (GRBV; *Grablovirus*, *Geminiviridae*) in grapevines [62].

2.2.4. Immunocapture-PCR (IC-PCR)

In this technique, viral particles are captured with virus-specific antibodies, followed by the release of viral nucleic acid for PCR amplification. This is very convenient for the detection of plant viruses in which inhibitory plant compounds or low viral titer hinder PCR amplification [63][64]. It has been successfully applied for the detection of BSMYV, which otherwise might lead to false positives with conventional PCR since there are integrated virus sequences in the host genome [65]. In addition to the ability to amplify the episomal viral sequences, IC-PCR also has the ability to concentrate virus particles from crude sap, thus making it more sensitive. IC-PCR is used for routine indexing of *BSV* worldwide [65][66]. It has also been useful for PRSV detection in leaf extracts of papaya and various cucurbits up to 1/10,000 dilution [67], and especially IC-PCR has been developed for GLRaV-3 detection in grapevines [68].

2.2.5. Loop-Mediated Isothermal Amplification (LAMP)

The PCR has been the main tool for viral detection; however, because of temperature cycling, it is time-consuming and less useful than isothermal methods. The LAMP technique has been developed for specific, sensitive, and rapid nucleic acid amplification. This assay uses a group of 4–6 special primers, which together with the strand-displacement activity of *Bacillus subtilis*-derived (Bst) NA polymerase, produce amplicons containing loop regions to which further primers bind, allowing amplification to proceed without thermal cycling [69]. The whole process can be carried out in 1 h at 60–65 °C in a heating block or water bath. Additionally, a pair of loop primers may or may not be used in the reaction. This assay is very useful in high throughput reactions with increased sensitivity and reduced amplification time [9]. LAMP products can be detected by conventional agarose gel electrophoresis or visual observation to estimate turbidity or color changes [70]. There are different LAMP methods for pathogenic microorganisms [71]. *Real-time LAMP* is a constant temperature amplification method carried out at 60–65 °C, for which only a simple water bath is required. This technique eliminates reverse transcription steps, as well as PCR instrument cooling time, which shortens the amplification time. Adding a fluorescent DNA intercalating dye into the reaction enables monitoring of a fluorescence amplification curve. Compared to conventional LAMP assays, this method avoids visible error, enables quantitative detection, and is more suitable for multi-sample analysis. *Reverse*

transcription LAMP can synthesize cDNA from template RNA and apply LAMP technology to amplify and detect them. As the template is an RNA sample, in addition to the reagents of DNA amplification, reverse transcriptase is added to the reaction mixture. After mixing and incubating at a constant temperature between 60–65 °C, amplification and detection can be carried out in a single step. *Multiplex LAMP* consists in simultaneous detection of multiple target genes in LAMP reaction, which increases diagnosis specificity. In addition, reverse transcription LAMP in a single tube can be coupled to multiplex LAMP for detection. *Electric LAMP* is based on an electronic simulation that provides fast and inexpensive putative tests of LAMP primers on target sequences compatibility. This aids to determine the opportunity of existing primers to detect recently discovered sequence variants. Finally, *in-disc LAMP* is an integrated device composed of micro-reactors embedded onto compact discs for real-time targeted DNA determination. This method requires similar reagents used in conventional LAMP, and it is performed in a micro-reactor placed in a 65 °C oven. During incubation, the disc is cyclically scanned and optically read to obtain quantitative results. LAMP has been developed and standardized for some of the viruses infecting bananas (BSV, CMV, BBTV and BBrMV), citrus (CYMV), grapevines (GLRaV-3), and apples (ASGV) [72][73][74][75][76][77][78][79]. The LAMP primers can be easily designed using software programs available, such as the PrimerExplorer V5 program [80]. This program is based on six regions in the target sequence, located on the right from the 5′ end and named F3, F2, F1, B1, B2, and B3. The program picks four LAMP primers: forward inner primer (FIP), backward inner primer (BIP), F3, and B3 primers. If needed, loop primer forward (LF) and backward (LB) are designed using the primer information file of the selected LAMP primers. The FIP consists of the F2 sequence at its 3′ end and the same sequence of the F1c region at its 5′ end. The BIP consists of the B2 sequence at its 3′ end and the B1c sequence at its 5′ end. Furthermore, the LF is designed using the complementary strand between F1 and F2 regions, while the LB is designed using the complementary strand between B1 and B2. In addition, the program takes into account four key factors: Tm, stability at the end of each primer, GC content, and secondary structures.

2.2.6. Recombinase Polymerase Amplification (RPA)

In this technique, the isothermal amplification of specific DNA targets is achieved by the combination of proteins and enzymes, such as recombinase, single-stranded binding proteins, and the strand-displacing activity of the polymerase. In combination, all these proteins produce amplicons in 10–15 min at a constant low temperature. There is no need for initial denaturation of the dsDNA target. RPA amplicons can be visualized on gel or by fluorescence and/or hybridization [81][82]. Compared to conventional PCR, which takes ~3 h for analysis, RPA analysis can be completed in just 1 h. Even though the cost of RPA reagents is much higher than in conventional PCR, the overall cost of the assay is reduced because PCR requires expensive thermal cyclers and purification of DNA using commercial kits. RPA is superior to other amplification techniques such as LAMP, which requires larger sets of primers, higher incubation temperature, purified DNA template, and longer incubation time [83], as RPA does not require a purified DNA template and can be easily performed using a very small amount of crude sap extract. Because of its simplicity, sensitivity, and quickness, it is an ideal technique for large scale plant virus indexing. Thus, RPA has been useful for BBTV diagnosis [83], as well as several other viruses such as little cherry virus 2 (LChV-2; *Ampelovirus*, *Closteroviridae*), PPV, tomato mottle virus (ToMoV; *Begomovirus*, *Geminiviridae*), and TYLCV, infecting fruit plant crops [81][82][84].

2.2.7. Rolling Circle Amplification (RCA)

RCA is another isothermal amplification method for viral detection in fruit plants. It uses exo-resistant random hexamer primers and the strand displacement activity of Phi29 DNA polymerase [85] to amplify circular nucleic acids. This technique was first applied for papillomaviruses diagnosis [86] and shortly after for geminivirus infections in tomatoes [87]. RCA is a sequence-independent amplification method carried out at isothermal temperature (30 °C). As random hexamers are employed, the prior sequence information of the viral genomes is not required, and it has the potential to amplify novel circular viral genomes. RCA followed by restriction fragment length polymorphism analysis has been used for the diagnosis of geminiviruses, which have small single-stranded circular DNA genomes [88]. However, the RCA product needs to be sequenced for confirmation of viral origin. RCA has been used even to amplify the bigger viral genomes of badnaviruses infecting bananas, which typically amplify the episomal viral genomes [89]. By employing this strategy, novel badnaviruses associated with the leaf streak disease of bananas have been identified, such as banana streak UA virus, banana streak UI virus, banana streak UL virus, banana streak UM virus, and banana streak IM virus (BSUAV, BSUIV, BSULV, BSUMV; *Badnavirus*, *Caulimoviridae*) [90]. Furthermore, random primed RCA has also been employed to identify the shorter banana streak OL virus (BSOLV; *Badnavirus*, *Caulimoviridae*) variants causing the leaf streak disease of bananas in India [91]. RCA, however, has some limitations: the amplification efficiency decreases with the length of the DNA template, and it is not suitable for larger genomes. Additionally, the probability of strand breaks increases with the length of the DNA molecule, resulting in the termination of the RCA. Furthermore, products generated from complex samples have to be analyzed further to exclude non-specific amplification [85]. RCA had primarily been used to detect plant viruses with small genomes (<3 kb) belonging to the families Geminiviridae and Nanoviridae [88][92]. However, by using a mixture of degenerate primers during the RCA, it was possible to detect plant viruses with larger genomes such as the *Badnaviruses*, *BSV* and sugarcane bacilliform virus (*SCBV*; *Badnavirus*, *Caulimoviridae*), and the *Caulimovirus*, cauliflower mosaic virus (CaMV; *Caulimoviridae*) [90].

2.2.8. Microarray

Some fruit plant crops are infected by a large number of viruses, as is the case of grapevine infected with more than 80 viruses. In such cases, techniques like ELISA and PCR are limited. In that sense, the development and application of DNA microarrays offer a convenient solution. Although microarrays were originally designed for simultaneous analysis of large-scale gene expression based on complementary base-pairing between the fluorescently labeled target sequences and the spotted probes on a solid surface [93][94], now they are used to detect thousands of plant and animal viruses in a single assay [95][96]. Initially, arrays for plant viruses were developed for the detection of viruses infecting a single crop or few families of plant viruses and viroids [97][98][99][100][101]. Moreover, since synthetic oligonucleotide probes provide greater sensitivity in detection, microarrays containing such probes enabled differentiation among different subgroups (or variants) of CMV and six potato viruses [98][102]. Another microarray containing 150 probes detected 49 viruses, including CMV and other viruses infecting non-fruit plants [103]. Additionally, three *Closteroviridae* members, including GLRaV-4, GLRaV-7, and GLRaV-9, were detected for the first time in Chilean grapevines using an oligonucleotide microarray [101]. A large-scale oligonucleotide microarray developed to identify 538 plant viruses detected CMV, TBSV, TSWV, and MNSV

[104]. Besides, it was reported the largest published crop-specific macroarray for the detection of 38 of the most prevalent or emergent viruses infecting grapevine [105]. This array contains 1578 virus-specific 60–70-mer oligonucleotide probes. In a survey of 99 grapevines from the United States and Europe, virus infections were detected in 46 selections of *V. vinifera*, *V. labrusca*, and interspecific hybrids. The majority of infected vines was singly infected, while some were mixed-infected with viruses from two or more families. Representatives of the four main virus families including *Betaflexiviridae*, *Closteroviridae*, *Secoviridae*, and *Tymoviridae* were found alone and in combination [105]. The main limitations of this technique are the high cost of spotting, need for labeled nucleotides, need for dust-free rooms, and little flexibility for use in differentiating strains, as well as the time for processing data [106].

2.2.9. Next-Generation Sequencing (NGS)

NGS technologies, developed in 2005, are massively parallel sequencing platforms that have allowed the rapid identification of viruses and viroids. Today, Illumina technology is the most widely used for sequencing. The process begins with DNA fragmentation and incorporation of adapters that contain segments acting as reference points during amplification, sequencing, and analysis [107]. With this technology, thousands of places throughout the genome are sequenced at once via massive parallel sequencing. NGS technology has made possible the direct identification of viruses and discovery of novel viruses in plants without antibodies or prior knowledge of viral sequences [108][109][110][111]. In response to viral infection, the plant produces small interfering RNAs (siRNAs), complementary to the viral genomic sequences that trigger degradation of viral RNAs in a process known as silencing [112]. Deep sequencing of siRNAs isolated from infected samples allows the recovery of either full or partial genomic viral sequences [109]. These methods have been useful to detect and discover viral infections in many horticultural crops [9][113]. NGS, in addition to its applications in resolving the etiology of viral diseases, characterization, and population genetics, has potential in the high-throughput diagnosis of plant viruses in plant crops [110][113]. The siRNA-based NGS parallel sequencing of symptomatic and asymptomatic samples followed by de novo assembly of long reads has the potential to identify the novel uncharacterized RNA, ssDNA, reverse-transcribing dsDNA viruses, and viroids without prior knowledge of the sequence [109][114]. In addition, conserved domains of viral groups have been identified for different genera or families, which enables designing primers targeting the regions that have the potential to identify different variants and new viruses [114][115]. Several methods to enrich the viral/viroid sequences in a total RNA pool [116], as well as algorithms for the identification of virus/viroid specific nucleic acids, have been developed [117]. In a short period of time, analysis of plant samples by NGS and homology-dependent computational algorithms have identified two new viroids and 49 new viruses from 20 known families [117]. More importantly, the development of user-friendly algorithms for handling voluminous NGS data for viral identification is challenging; however, once optimized to analyze a large number of samples, NGS diagnostics can be used as a reliable tool for certification of horticultural plants destined for exportation. Finally, indexing of the mother plant by NGS, which is used for in vitro large-scale multiplication of crops, can avoid vertical propagation of viral diseases that are a major problem in bananas, citrus and passion fruits.

2.3. Biosensors

Biosensors are portable diagnostic devices based on antigen–antibody interactions (immunosensors) or nucleic acid hybridization coupled to a physicochemical transducer microsystem [118]. A biosensor is made up of a receptor, a transducer, and a processor, thereby making this technology economic and highly sensitive for immediate viral detection from leaf extracts. Transducers are classified according to the parameters of measurement as optical (detecting changes in light transmission), thermometric (measuring temperature changes), potentiometric (measuring potential at constant current), amperometric (measuring current at constant potential), cyclic voltametric (measuring current at variable potential), magnetic, or piezoelectric (measuring changes in mass) microsystems. These transducers convert the biological signals into electrical signals of intensity directly proportional to the concentration of a specific analyte [119]. Because of their large surface area, high biocompatibility, and high electron transfer potential, the gold nanoparticles (GNPs) are used to immobilize various biomolecules.

The biosensor based on the bioelectric recognition assay (BERA) is an intelligent system for the detection of plant viruses, combining the principle of artificial neural networks and biosensors. For example, BERA biosensors detect the electric response produced by the interaction of a cell culture suspended in a gel matrix with the CGMMV [120]. This response was indirectly generated, taking into consideration the signal produced by antibody recognition of CGMMV CP. A biosensor based on magnetic immunoassay was developed for the detection and quantification of GFLV, which was achieved through a double-antibody sandwich immunofiltration approach [121]. Additionally, an amperometric biosensor was developed for capsicum chlorosis virus (CaCV; *Orthotospovirus*, *Tospoviridae*) diagnosis, which showed ~1000 times more sensitivity than DAC-ELISA [122]. The antigen sample was placed onto the surface of GNP/multiwalled carbon nanotube screen-printed electrodes in order to interact with polyclonal antibodies specific for CaCV and GBNV. The quartz crystal microbalance (QCM) is a sensitive mass-measuring device consisting of a quartz crystal wafer sandwiched between two metal electrodes connected to an external oscillator circuit that records the resonance frequency [123]. Piezoelectric immunosensors based on artificial or natural antibodies and QCM are able to detect TMV and maize chlorotic mottle virus (MCMV; *Machlomovirus*, *Tombusviridae*), and they could be useful to detect viruses infecting fruit plants [124][125].

An optical miniaturized paper-based DNA sensor identified the early infection caused by BBTV. Some DNA biosensors are based on nucleic acid hybridization and QCM detection of DNA molecules [126][127]. In this regard, the nucleic acid-based QCM biosensors developed to detect cymbidium mosaic virus (CymMV; Potexvirus, *Alphaflexiviridae*) and odontoglossum ringspot virus (ORSV; *Tobamovirus*, *Virgaviridae*) infections in orchids are more sensitive than those based on antibody recognition [128]. Compared to antibodies binding to CP in QCM immunosensors, QCM DNA biosensors possess immobilized nucleic acid sequences that bind more efficiently with their complementary virus CP gene sequences [128]. A potentiometric biosensor was used for the detection of DNA sequences from PPV in plant extracts [129]. Additionally, a cyclic voltametric biosensor was able to differentiate sugarcane white leaf phytoplasma and sugarcane mosaic virus (SCMV; *Potyvirus*, *Potyviridae*) infections [130].

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