International Journal of Plant & Soil Science

Volume 35, Issue 22, Page 339-350, 2023; Article no.IJPSS.109590
ISSN: 2320-7035

Revolutionizing Plant Virus Detection: A Review on Molecular Breakthroughs and their Implications

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2023/v35i224142

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/109590

Review Article

Received: 14/09/2023
Accepted: 20/11/2023
Published: 23/11/2023

ABSTRACT

Viruses are very minute microorganisms which are incapable of independent existence and can infect wide range of wild and cultivated plants. The plants' reaction to viral infections are diverse and depends on factors such as the specific virus- host interaction under different environmental conditions with symptoms ranging from acting as asymptomatic carrier to showing severe diseases symptoms which further results in plant mortality. Since various viral infections can result in similar symptoms, relying solely on the disease's external appearance offers only partial insights for disease diagnosis. To achieve more precise and dependable virus identification, it is essential to consider various characteristics and properties of the virus. The molecular detection of plant

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viruses has revolutionized the field of plant virology. This review highlights the overview of the different approaches utilized in the diagnosis of plant viruses, with a particular focus on significant advancements in molecular techniques, ranging from traditional PCR-based methods, ELISA to high-throughput Next-Generation Sequencing (NGS) technologies. Molecular tools have enabled precise and rapid identification of plant viruses, contributing to better disease management in agriculture. The review also delves in to emerging technologies such as CRISPR- based diagnostics and nanopore sequencing, which hold promise for enhancing the precision and speed of plant virus detection.

Keywords: PCR; ELISA; NGS (Next- Generation Sequencing); CRISPR; Nanopore sequencing.

1. INTRODUCTION

Plant viruses are very small size pathogenic entities and hence cannot be observed through light microscopes. They necessitate the use of transmission electron microscopes. Viruses are structured with a protective coat protein called capsid enveloping a core that carries genetic information in the form of either DNA or RNA. Their genome size is very small which encodes only few proteins hence it's very difficult to trace virus and control them [1]. Viral infections in crops have a detrimental impact on crop development, leading to diminished yields, affecting the survival of grafts or scions, and compromising the quality of fruits causing significant economic losses [2]. Viruses induce a range of symptoms in infected plants, including mosaic patterns, yellowing, chlorosis (loss of green color), virescence (abnormal growth of chlorophyll), fasciation (abnormal flattening of plant parts), fasciculation (formation of abnormal clusters), stunting, witches' broom-like growth, and necrosis (cell death). These symptoms result in impaired growth and reproductive functions, ultimately causing a decline in the overall health and productivity of the plants [3].

Identifying viral diseases in plants based on symptoms can be quite challenging as some plant viruses can infect plants without manifesting visible signs, making symptom-based diagnosis more complicated. Accurate detection using precise and sensitive techniques plays a vital role in the effective management of plant diseases. Additionally, the presence of specialized tools for virus diagnosis and identification is a fundamental requirement for the development and assessment of disease management strategies [4].

Due to wide diversity in genetic material of viruses infecting plants and their high mutation rates, it's very difficult to fully rely on any single method of detection. Hence, all methods starting from preliminary visual observation to microscopic identification serological methods, molecular methods, and next generation sequencing must be employed to allow early identification and management of virus. These tests allow for the exact identification of viral pathogens in plants, guaranteeing a more precise diagnosis [5]. With advancement in scientific field and introduction of next-generation sequencing (NGS) technologies, millions or even billions of nucleotides can be sequenced simultaneously without need of prior sequence orientation leading to significant transformation in the field of plant virus diagnosis [6]. Comprehensive discussions of key diagnostic methodologies, ranging from conventional visual assessments of indicator plants to cutting-edge third-generation sequencing technologies, are outlined in the following sections.

2. CONVENTIONAL MORPHOLOGICAL ASSESSMENT OF DISEASED PLANTS

Historically, the predominant method for identifying plant pathogens involved visually inspecting infected plants and seeds based on specific symptoms, using indicator plants, assessing their host range, examining physical characteristics of virus such as their shape and size, and studying virus -vector relationships. When the symptoms are distinct and easily recognizable, visual inspection is a straightforward task. Nonetheless, several variables like the virus strain, the specific plant variety, the timing of infection, and environmental conditions can all impact the appearance of these symptoms [7]. When dealing with a plant viral disease of established origin, the key factor in its recognition typically hinges on the symptoms evident in the host plants as it bears a distinct relation to the disease. The external indications of viral plant infections are intricately linked to irregularities in plant physiology, and these symptoms are further classified into mosaics and chlorosis [8]. Visual inspections can

be carried out using various techniques, including visible-light imaging, chlorophyll fluorescence imaging, hyperspectral imaging, and thermal imaging [9]. In most cases, it is important to combine visual symptom inspection in the field with additional confirmatory tests to ensure a precise diagnosis of viral infection as in plants, to get clarity in mixed infections, in different environmental or growing conditions, and depending on species or cultivar [10].

2.1 A Close-up View of the Invisible World of Virus through Microscopy

Understanding the biology of viruses and the causes of viral diseases is essential for preventing these diseases, ensuring accurate and effective virus diagnosis, and implementing virus control measures. A conventional method for studying viruses in plant tissues entails using advanced light microscopy and high-resolution electron microscopy, enabling the visual inspection of viruses within plants. The exceptional ability of Electron Microscopy (EM) to resolve at the nanometer scale allows for the direct visualization of viruses, offering valuable images for diagnostic and research purposes. Transmission Electron Microscopy (TEM) has advanced the characterization of viral particle morphology in both unprocessed and purified samples. These TEM studies have led to one of the earliest proposals for classifying viruses based on their distinctive morphological and serological associations, along with certain biological features. TEM enables direct detection, involving the initial homogenization of infected tissue followed by the application of negative staining. TEM's defining characteristic is its ability to offer an expansive and immediate view, enabling the rapid assessment of the current situation, including the quantity and configuration of viruses present, and even revealing unexpected findings [11]. As an initial stage in identifying pathogens, TEM necessitates only small sample quantities that contain a significant viral load. TEM is impartial towards RNA or DNA genomes because it focuses on the input of proteins, the viral capsid, or ribonucleoprotein (RNP) complexes [12]. Tobacco mosaic virus is known for its characteristic production of needle-shaped and hexagonal crystals, whereas Potato virus X and wheat mosaic virus are notable for their capacity to generate spherical structures that do not crystallize. Various viruses that affect plants, including Tomato yellow leaf curl disease (TYLCD), Potato virus S (PVS), Rice stripe mosaic virus (RSMV), Tomato brown rugose fruit virus (ToBRFV), Pepino mosaic virus (PepMV), and Potato virus M were investigated and studied using electron microscopy techniques [13-16]. Therefore, TEM acts as a crucial instrument for establishing which of the available techniques such as bioassays, serological methods, or molecular biology approaches should be employed next to identify the genus and species of the virus more precisely.

3. PROBING THE PHYSICAL FEATURES OF VIRUSES

In the past, characteristics of a virus like its thermal inactivation point, dilution end point, and in vitro longevity were utilized as indicators of the virus's infectivity in sap extracts and for identifying plant viruses. Nevertheless, these properties have been found to be unreliable and are no longer recommended for virus diagnosis though still studied in literature [17].

4. SEROLOGICAL ASSAYS

Serological tests are based on the interaction of specific antibody with antigen against which it is produced. One such method based on serological assay is ELISA. Traditional ELISA procedures involve the use of polystyrene plates that can bind antibodies or proteins and incorporate an enzyme-substrate reaction. The application of enzyme-linked immunosorbent assay (ELISA) is highly valuable tool for virus detection in plants. The techniques, such as ELISA (Enzyme-Linked Immunosorbent Assay), which rely on the dependable detection of protein molecules using either polyclonal or monoclonal antibodies, are extensively utilized in the realm of plant viral diagnostics. Clark and Adams were the first to employ the ELISA method for the diagnosis of plant viral diseases. The ELISA technique's practical efficiency remained consistent, irrespective of any ratio between antibodies and antigens. Once the appropriate concentrations were established, they could be universally applied for subsequent virus detection tests, regardless of the virus concentration. The enzyme-labeled antibody's reaction was directly linked to the virus concentration, underscoring the technique's robust quantitative potential. Bar-Joseph et al. [18] conducted experiments to investigate the serological reactions of four different plant viruses, namely citrus tristeza virus (CTV), carnation mottle virus (CarMV), carnation yellow fleck virus (CYFV), and tobacco mosaic virus (TMV). They analyzed how these viruses interacted with their corresponding gamma-

globulin alkaline phosphatase conjugates when applied in a sandwich format on antibody-coated microplates. Among ELISA-based approaches, direct tissue blot immunoassay (DTBIA), double antibody sandwich (DAS) ELISA, and tissue-print (TP) ELISA stand out as the most employed methods for viral identification [19-21]. Numerous variations of ELISA have been developed and applied to enable the swift detection of various plant viral diseases in a diverse range of host plants.

4.1 The Dot Blot Immunobinding Assay (DBIA)

It is a modified blotting technique widely employed to specifically identify nucleic acids and proteins. In this method, antigen is applied onto a nitrocellulose membrane in the form of drop of a test sample. Then the test antibody is added to nitrocellulose membrane, followed by exposure to a peroxidase-conjugated secondary antibody targeting the first antibody. The final step involves the development of the assay using 4-chloro-1-naphthol. This DBIA procedure is utilized for screening the supernatants of hybridomas to detect monoclonal antibodies and to screen pathological sera for multiple antibodies [22]. This technique can detect viruses even if very small quantity of antigen or crude sap is available.

4.2 Double Antibody Sandwich (DAS) ELISA

It is a form of indirect enzyme-linked immunosorbent assay (ELISA) that has been created for the purpose of detecting viruses in plants. This technique involves using protein in two stages to form a sandwich-like structure with layers of antibody-antigen-antibody. The initial application of protein primes the microtiter plate for the antibody coating layer. The second layer of protein is conjugated to the enzyme and is responsible for identifying the second antibody layer by giving a particular colour on reaction with substrate.

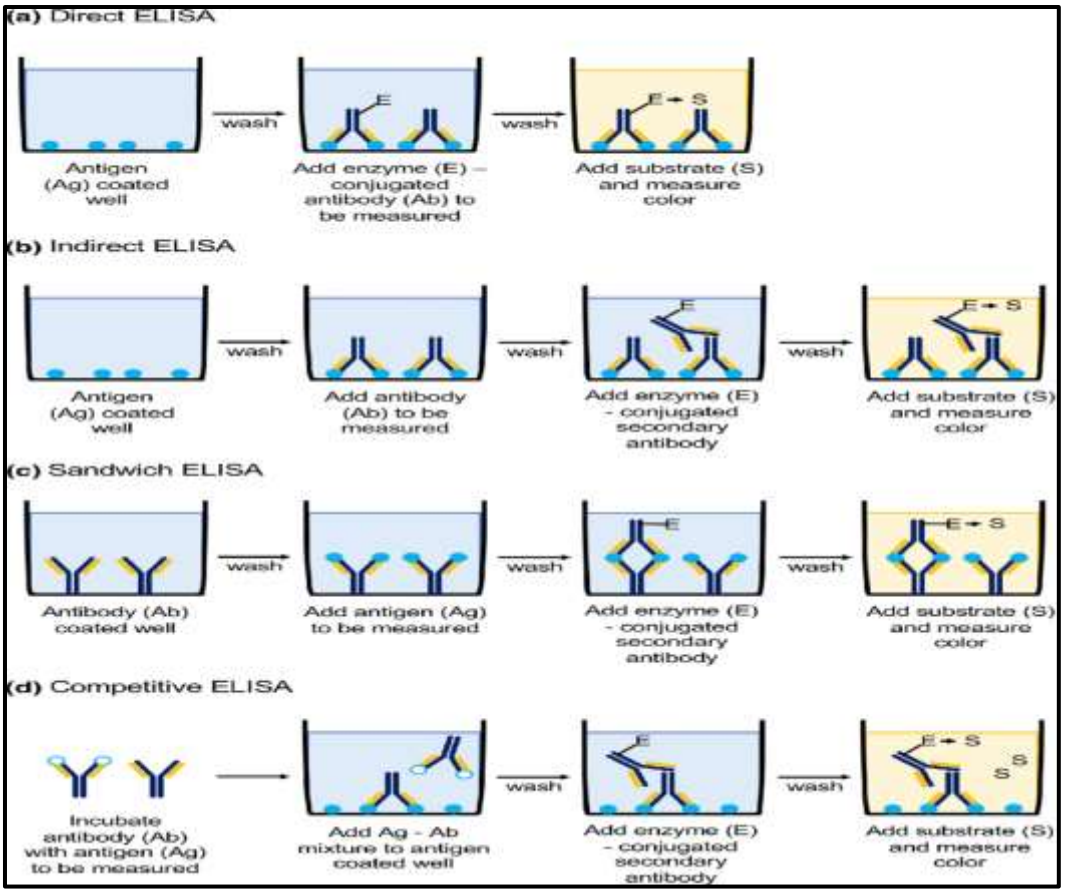

Fig. 1. Schematic presentation of basic types of ELISA (enzyme-linked immunosorbent assay): a direct, b indirect, c sandwich, d competitive; Ag antigen, Ab antibody, E enzyme, S substrate
(Source: Research Gate)

4.3 Tissue Blotting Immunoassay (TBIA)

Tissue blots were created gently but firmly by pressing freshly cut plant tissue onto nitrocellulose membranes. Infected freshly cut plant tissue is directly electro-blotted on to the nitrocellulose membrane (transfer of viral antigen to membrane). The existing antigens were first reacted with primary antibodies specific to virus antigen and detected with enzyme labelled secondary antibodies also called probes that react with primary antibodies [23]. Distinct reactions specific to the Faba bean necrotic yellow virus (FBNYV) antigen were noticed on tissue blots from infected plants, whereas no such reactions were observed on tissue blots from non-inoculated faba bean plants. The red staining was localized to the midrib and secondary vein regions of leaf, petiole, and stem sections, indicating that the FBNYV was confined to the vascular tissue [24].

ELISA relies on the interaction between antibodies and antigens, highlighting the critical need for highly specific antibodies that can accurately detect the target substance. However, ELISA may occasionally produce incorrect positive results, mainly because of non-specific responses or the cross-reaction with specific components found in the samples [25]. In summary, although ELISA has been widely employed in diagnostics, its shortcomings have become more apparent. Hence, we need to discuss nucleic acid-based methods, CRISPR based technologies and high-throughput next-generation sequencing (NGS) technologies.

5. NUCLEIC ACID-BASED METHODS

Nucleic acid-based methods have brought a significant transformation to the realm of viral detection. They provide excellent sensitivity and precision while enabling the identification and detailed analysis of various viruses, even including newly emerging ones. The selection of a particular technique relies on factors such as the specific virus under investigation, the type of sample, available resources, and the desired speed in obtaining results. The rapid advancements in molecular and genomics technologies have expanded the applications of these methods, particularly in the detection of infectious pathogens such as viruses. Due to their extreme higher sensitivity, nucleic acid-based techniques whether focused on DNA or RNA, have gained widespread acceptance in viral diagnostics. In general, nucleic acid-based analysis involves three crucial stages: nucleic acid extraction (either DNA or RNA), amplification, and the pivotal step of product analysis, which directly provides the test result [15]. Amplification can be achieved by various PCR techniques which are discussed below and further sequencing can be carried out for virus detection.

5.1 PCR (Polymerase Chain Reaction)

DNA amplification techniques for viral detection can be analyzed with polymerase chain reaction (PCR). PCR technology has made substantial strides in simplifying and enhancing the detection and diagnosis of specific genes. This technique involves creating millions of copies of a specific region of the viral genome, which can be visualized through methods like electrophoresis or by using fluorescent probes. PCR can utilize genomic DNA or complementary DNA generated through reverse transcription (RT) of viral RNA as templates. The amplification process consists of three major steps which are denaturation, annealing followed by extension. These three steps are repeated through multiple cycles (usually 20 to 40 cycles), with the newly synthesized DNA segments serving as templates for subsequent cycles [26].

5.1.1 Real-time quantitative PCR (qPCR)

It is a modified version of PCR method that tracks the advancement of the reaction by identifying a fluorescent marker that attaches to the double-stranded DNA or gets released from specific 15 to 30 nucleotide probes. This form of PCR is employed for the purpose of measuring nucleic acids accurately. Further, isothermal DNA amplification can be achieved by various methods such as i) Helicase dependent amplification (HAD) ii) Recombinase polymerase amplification (RPA) iii) Nucleic acid sequence-based amplification method (NASBA) iv) Loop-mediated isothermal amplification (LAMP) [27]. More recently, novel molecular diagnostic tools have been created, utilizing the prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) immune system, which is widely recognized for its applications in genome editing [28]. The PCR technique is employed to oversee the effectiveness of antiviral treatment for HIV-1, HBV, HSV-1, and HSV-2. Performing uniplex RT-PCR for each virus or viroid separately can be costly and demanding in terms of resources, as it necessitates both time and materials.

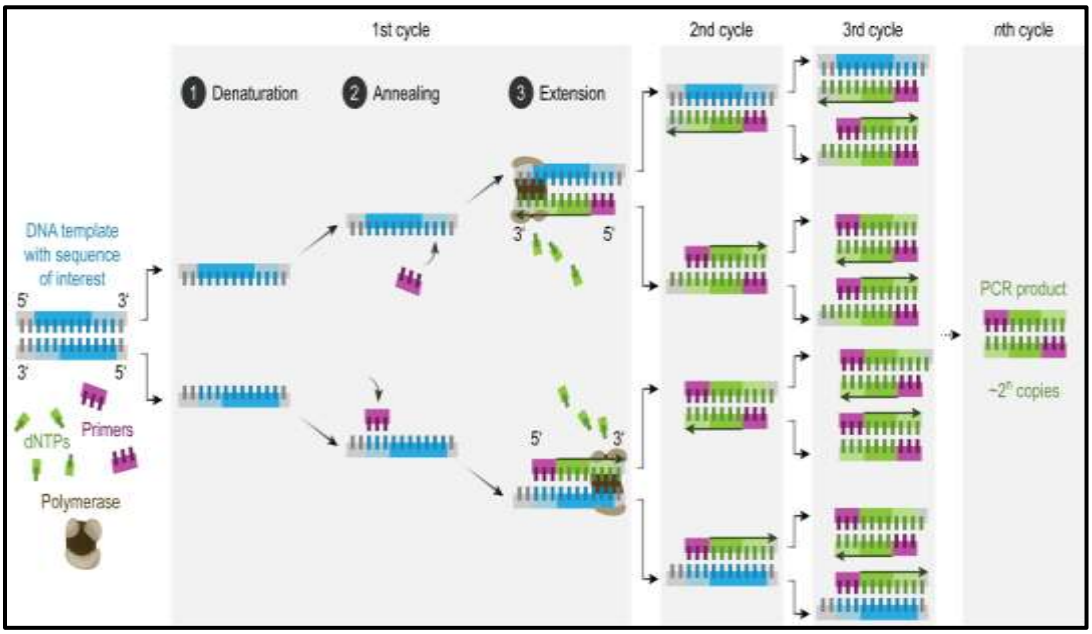

Fig. 2. Schematic diagram of Polymerase Chain Reaction
(Source: Wikipedia)

5.1.2 Nested-PCR

It is a method within the realm of polymerase chain reaction (PCR) that enhances both the sensitivity and specificity of DNA amplification. This approach employs two distinct sets of amplification primers and involves a two-step amplification process. In the initial round, a single pair of primers is employed for a specified number of cycles, typically ranging from 15 to 30. The resulting amplified product from the first round serves as the template for a subsequent round of amplification, utilizing a different pair of primers. However, it is noteworthy that a notable drawback associated with nested-PCR is the increased susceptibility to contamination issues.

5.1.3 Multiplex PCR

Multiplex PCR for DNA targets and multiplex RT-PCR (mRT-PCR) for RNA targets are efficient, dependable, and cost-effective techniques that have proven effective in simultaneously identifying various pathogens in a single test. With mRT-PCR, distinct sets of specific primers are used for two or more targets within a single reaction tube, allowing for the concurrent amplification of multiple target nucleic acids in a single assay. Multiplex detection of more than three targets by fluorescent probes is carried out by the dyes with compatible range of spectra. Simultaneous detection of four pathogens has been reported for four retroviruses [29].

6. DNA MICROARRAYS

DNA microarrays or biochips consist of a surface to which multiple capture probes are attached, with each probe designed to be specific for a DNA or RNA sequence of the target. Their primary function is to detect numerous sequences in a single test. Various materials are currently used to create microarrays, including glass, nylon, and different types of polymers. A single chip can accommodate up to 30,000 DNA probes, representing gene sequences. These probes can be PCR products that have been amplified to high concentrations or relatively short oligonucleotide probes, typically ranging from 30 to 50 base pairs. Once the probes are arrayed on the chip, it can be exposed to DNA/RNA from the sample to be tested, which is labeled with fluorescent markers [30].

7. LOOP MEDIATED ISOTHERMAL AMPLIFICATION

The LAMP method is a simple and easily executable technique, provided that the suitable primers have been prepared in advance. This process necessitates the utilization of a DNA polymerase, four primers, and a conventional laboratory water bath or heat block for the reaction. LAMP amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions. The year 2000 marked the

inception of Loop-mediated isothermal amplification (LAMP) technology, with the primary goal of improving the efficiency of nucleic acid amplification in relation to sensitivity and specificity [31].

A combination of Loop-mediated isothermal amplification (LAMP) and subsequent detection using a real-time fluorescence assay with CRISPR-Cas12a was employed to identify the presence of tomato yellow leaf curl virus and tomato leaf curl New Delhi virus [32].

8. CRISPR/Cas

CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated) is an innate immune system naturally employed by prokaryotic organisms like bacteria and archaea to protect themselves from invasive viruses and plasmids 33,34]. CRISPR/Cas has become a popular choice for genome editing because of its precise targeting and the ability to modify any gene by changing the guide RNA sequence. Its adaptability for specific targets has led to its use in interfering with viruses in a range of organisms, including plants [35,36].

9. IN SITU HYBRIDIZATION (ISH)

In situ hybridization is a technique used to identify and pinpoint a particular genetic sequence within a single cell. This genetic sequence is specifically attached to a tissue sample through matching base pairs, or hybridization, with a visible genetic fragment known as a probe. In various scientific domains, including virus exploration, in situ hybridization (ISH) is commonly employed. The advancement of high-throughput techniques such as next-generation sequencing has led to a higher identification of novel viruses. ISH proves to be a valuable tool in verifying a potential link between a recently identified pathogen and changes in tissue [37,38].

10. HIGH-THROUGHPUT SEQUENCING

The introduction of high-throughput sequencing (HTS) methods, commonly referred to as next-generation sequencing, has brought about a significant transformation in the diagnosis of plant viruses [39,40]. High-throughput sequencing (HTS) does not require prior knowledge of viral genetic codes and can simultaneously sequence millions or even billions of DNA strands, allowing us to identify all the viruses in a plant [41].

High-throughput sequencing (HTS) can be categorized into two main types i.e., Second-generation sequencing and third generation sequencing. The second-generation sequencing relies on the generation of random libraries of DNA fragments using the initial DNA material or cDNA obtained through retro transcription of RNA with random primers or oligo-dT. These libraries are subsequently clonally amplified, attached to synthetic DNA adapters, and then sequenced in a parallel manner. This process generates a substantial number of short sequences reads, typically ranging from 100 to 500 nucleotides in length. These short reads are subsequently assembled by aligning overlapping sequences based on nucleotide identity, and this assembly is achieved through computational analysis, often using software tools like the geneious package. (www.geneious.com). Furthermore, high-throughput sequencing (HTS) is employed for investigating a broad spectrum of scientific inquiries in the realm of plant virology. These applications encompass the identification of novel plant viruses and viroids, epidemiological investigations, exploration of synergistic interactions among viruses, and the analysis of genetic diversity and the evolutionary mechanisms that drive virus populations [42-46].

Third-generation sequencing involves the direct, real-time sequencing of individual DNA molecules, eliminating the necessity for clonal amplification. This innovation not only streamlines the DNA preparation process but also yields extended reads spanning several kilobases in length. Goodwin et al. [47]; van Dijk et al. 2018).

Diverse biotechnological enterprises are actively engaged in the advancement of genomic sequencing methodologies, exemplified by innovations like Single-Molecule Real-Time (SMRT) sequencing and Nanopore sequencing. In the context of SMRT sequencing, it entails the utilization of a specialized flow cell replete with an array of myriad picolitre wells, each featuring a transparent base referred to as zero-mode waveguides. Within these wells, a DNA polymerase is immobilized. The sequencing process involves the continuous visualization of nucleotide incorporation into individual DNA molecules ensconced in the wells. A laser and camera system are employed to meticulously capture the emitted light's color and duration as nucleotides are temporarily held in abeyance at the well's bottom during the incorporation event.

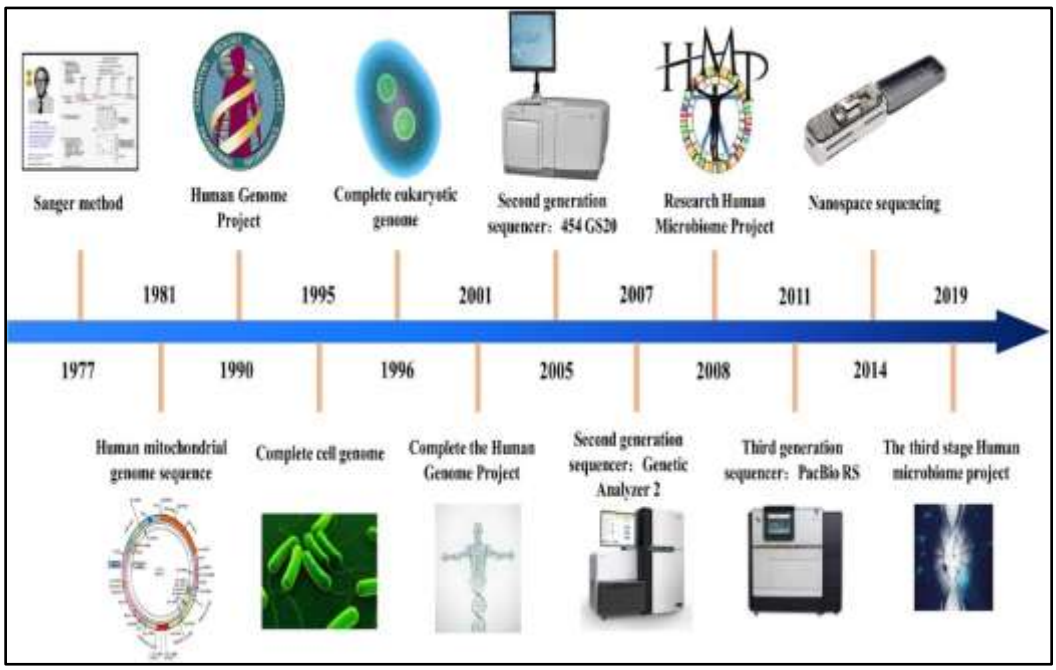

Fig. 3. History of next generation sequencing
(Source: Yang et al. [52]).

Conversely, Nanopore sequencing hinges upon the controlled translocation of DNA or RNA strands through a nanopore, which can manifest within membrane proteins or artificial materials such as silicon nitride and aluminum oxide. This passage induces an ionic current that is governed by an applied voltage. The unique aspect of Nanopore sequencing lies in its capability to discern variations in the ionic current, contingent upon the structural attributes, dimensions, and length of the traversing DNA sequences. This process endows Nanopore sequencing with several merits, including cost-efficiency relative to other High-Throughput Sequencing (HTS) technologies, portability due to the compact size of the sequencing apparatus, and expeditious sample processing. Furthermore, it obviates the necessity for reverse transcription when working with RNA viruses [48]. This cutting-edge technology has been employed in recent times for the identification of various plant viruses, such as plum pox virus (PPV), the tomato yellow leaf curl virus (TYLCV), as well as for the exploration and detection of previously unknown plant viruses [49-51].

However, next-generation sequencing generates vast amounts of data, and the analysis and interpretation of this data can be computationally demanding. Bioinformatics expertise is crucial for extracting meaningful information from the sequence data generated [53,54].

11. CONCLUSION

The molecular detection of plant viruses has become an essential and highly effective tool in the realms of plant pathology and agriculture. The application of advanced molecular techniques has revolutionized our capacity to precisely identify, characterize, and monitor plant viruses. The significance of molecular methods lies in their ability to detect viral pathogens at early infection stages, often preceding visible symptom manifestation. This early identification is critical for the timely implementation of disease management strategies, thereby minimizing yield losses and ensuring food security. Polymerase chain reaction (PCR)-based techniques, including reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR), have become standard for specific viral nucleic acid detection. These methods not only assure accurate identification but also enable quantification of viral load, aiding in the assessment of disease severity. Moreover, the introduction of innovative technologies like loop-mediated isothermal amplification (LAMP) and next-generation sequencing (NGS) has broadened our ability to detect various viral pathogens in a high-throughput manner. NGS, particularly, allows for a comprehensive analysis of viral communities in plant samples, offering insights into virus diversity and evolution. Beyond their diagnostic role, molecular detection methods significantly

contribute to plant virus research, providing valuable information on virus-host interactions, epidemiology, and the development of resistant crop varieties. These insights enhance our comprehension of plant-virus dynamics and inform the creation of sustainable disease management strategies and thereby have not only transformed our ability to monitor and manage plant viral diseases but also positioned us to safeguard global food production, promote sustainable agriculture, and contribute to the enduring resilience of plant ecosystems.

12. CURRENT CHALLENGES

Plant viruses exhibit high genetic variability, leading to the emergence of new strains and variants. This genetic diversity can pose challenges for the design of universal primers and probes for molecular assays. There is often a lack of standardized protocols for molecular diagnostic methods and advanced techniques such as next-generation sequencing, may not be readily accessible in all regions or to all researchers. Limited access to technology can hinder the widespread adoption of molecular methods, especially in resource-limited areas. Moreover, molecular assays can be susceptible to false positives or false negatives. Factors such as sample quality, contaminants, and inhibitors can impact the accuracy of results, emphasizing the need for rigorous quality control measures. The cost of equipment, reagents, and maintenance for molecular diagnostics can be a limiting factor, especially for smaller research labs and farmers in developing regions. Thus, while molecular diagnostic tools are highly specific and sensitive, it is essential to complement them with other methods, such as traditional culture-based techniques or emerging proteomic approaches, which hold promise in revealing insights into pathogenicity and virulence factors. This, in turn, broadens the possibilities for diagnosing and safeguarding crops against diseases.

13. FUTURE IMPLICATIONS

The advancements in molecular detection of plant viruses open several promising avenues for future research, with implications for both fundamental understanding and practical applications in agriculture. Hence, in order to strengthen our studies, we need to investigate the intricate molecular interactions between plant viruses and their host plants. It is very essential to explore the evolutionary dynamics of plant viruses, including the factors influencing the emergence of new viral variants. This research could aid in predicting and managing viral disease outbreaks, as well as in developing strategies to mitigate the impact of evolving viral populations on crop production. Further it is very important to work on metagenomic approaches, such as next-generation sequencing, to comprehensively characterize viral communities within plant ecosystems. This can provide insights into the diversity, distribution, and dynamics of plant viruses in different agroecosystems. There is need to work on standardizing and validating molecular detection protocols for a wider range of plant viruses. This is crucial for ensuring the reliability and comparability of results across different laboratories and regions, facilitating effective disease management on a global scale. At last, it is essential to develop and optimize rapid, point-of-care diagnostic tools based on molecular techniques. The scientists should investigate integrated disease management strategies that combine molecular detection with other approaches, such as cultural practices, biological control, and precision agriculture. This holistic approach can enhance the sustainability of crop production and reduce reliance on chemical interventions. Bridging the gap between research and practical application is crucial for the effective implementation of advanced technologies in diverse agricultural settings.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/109590