

Review

# Applicability of Smart Tools in Vegetable Disease Diagnostics

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**Abstract:** Various diseases and pests cause serious damage to vegetable crops during the growing season and after harvesting. Growers attempt to minimize losses by protecting their crops, starting with seed and seedling treatments and followed by monitoring their stands. In many cases, synthetic pesticide treatments are applied. Integrated pest management is currently being employed to minimize the impact of pesticides upon human health and the environment. Over the last few years, “smart” approaches have been developed and adopted in practice to predict, detect, and quantify phytopathogen occurrence and contamination. Our review assesses the currently available ready-to-use tools and methodologies that operate via visual estimation, the detection of proteins and DNA/RNA sequences, and the utilization of brand-new innovative approaches, highlighting the availability of solutions that can be used by growers during the process of diagnosing pathogens.

**Keywords:** phytopathogen; diagnostic; serology; PCR; LAMP; RPA; barcoding; NGS; CRISPR/Cas; vegetable



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## 1. Introduction

As a source of vitamins, minerals, and other nutrients, vegetables constitute an important food component that is produced and traded worldwide. As with other cultivated species, vegetables grow in tandem with beneficial or harmful microbiota that affect the yield and quality of crops. These microbiotas exist in fields, and the global movement of plant material (vegetable seeds, seedlings, and produce) provides opportunities for phytopathogens to cross borders and spread to new areas. It has been shown that the number of phytopathogens in different regions is associated with the volume of imports. In this way, the risk of pathogenic species expansion from one country to another has been increasing [1,2]. To prevent invasions, several control systems have been set up by national authorities, following the recommendations of scientists and international bodies [3]. Growers also try to prevent locally occurring plant pathogens from invading their crops, and they carefully monitor the possible spread of new phytopathogens from neighboring regions [4]. To successfully cope with vegetable pathogens, preventive measures should be taken, and consideration should be given to soil management, seed treatment, the cultivar’s natural resistance, and the various monitoring systems that are suited to conventional, biotechnological, and organic farming, forming part of the IPM recommendations [5]. The ability to capture the pathogen upon its first attack as early as possible, as in the early infection stage, allows growers to make appropriate and effective management decisions. It is apparent that tools for disease diagnosis and pathogen identification are greatly needed. Otherwise, it would not be possible to take preventative steps and set up management measures in cases of infection [6].

Several approaches and techniques have been developed to facilitate the diagnosis, monitoring, and study of phytopathogenic agents in plants and [7–9] various vegetable species. Some of these techniques have been validated and are used by accredited laboratories for official controls [6,10–12]. Others have significant potential for application in “smart” farming [7,9], possibly leading to the implementation of phytopathogen surveillance through intelligent and goal-oriented analysis, planning, and observation [13]. Currently, the importance of smart approaches for disease diagnosis on farms is increasing [14,15]. We aimed to map fully validated procedures employed in official controls and concurrently tools at various technological readiness level (TRL) that are usually verified in a single laboratory only [16] to determine their future potential as new devices for phytopathogen detection [16].

## 2. Symptomatic Diagnosis

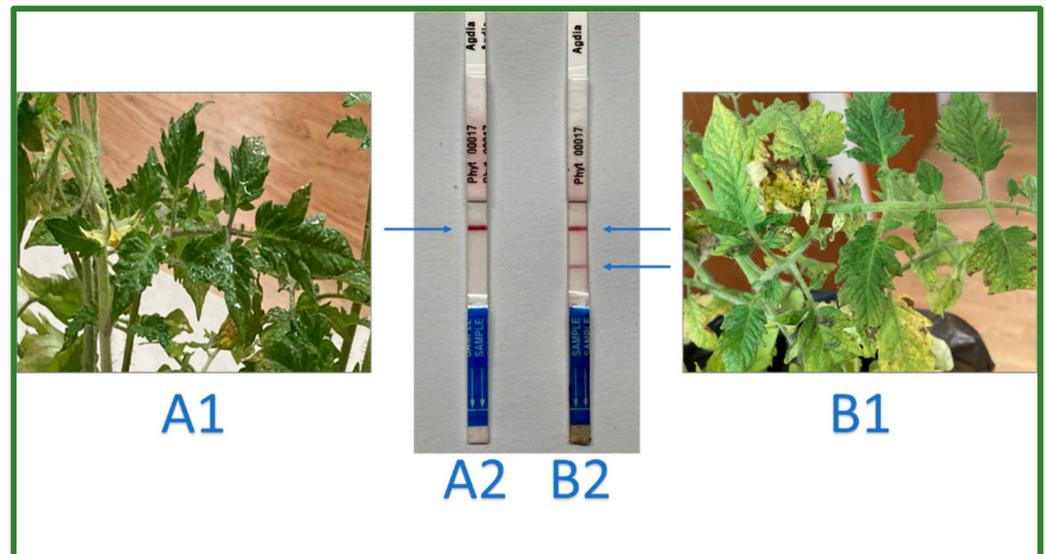
Symptomatic evaluation was once the sole option for disease identification in field crops. However, the symptoms of individual diseases, especially at the onset of their development, can be ambiguous. Therefore, this approach is based on experience and requires highly skilled specialists [17,18]. The organisms that cause plant diseases are very small (microscale) and can, therefore, only be detected by lay farmers based on the impacts of their attacks. As experts are not always available when needed, several options have been introduced and tested in efforts to improve the available diagnostic approaches. Mainly, these include simple phytopathogen cultivation tests [19], but these tests are time-consuming and dependent on mycological skills and laboratory infrastructure. Moreover, they are not sufficiently sensitive to enable detection in the early stages of infection. Previously, higher-level image analysis of crop diseases was not particularly effective; however, its applicability has been enhanced when combined with smart tools, such as videos or mobile phones linked to knowledge bases (e.g., the system VACDDS [20]). Various computational algorithms and diagnostic models have been tested to improve visual evaluation and further identification [21,22]. The existence of precise databases of sufficiently adequate images has been [23] reported to be the basic prerequisite for enabling such a system to be functional. These precise tools for phytopathogen interception are still expensive and, in the case of vegetables, not sufficiently advanced. Several factors, such as the unclear outlines of lesions, uneven coloring of leaves, and variability in lighting conditions, among others, hinder analysis [24]. The currently available image-based techniques have been reported to be precise under well-defined experimental conditions, but when they are moved to the field, higher precision and reliability are required. Thus, there exists a great demand for tools that can facilitate the precise detection of pathogens for individual crops.

## 3. Serological Tests

Serological tests are widely used in diagnostics. They work by exploiting the plurality of antibody reactions to pathogens. The enzyme immunoassay (EIA) is employed to detect the presence of a ligand, e.g., proteins in a liquid sample using antibodies directed against the protein to be measured. One modification, the enzyme-linked immunosorbent assay (ELISA) [25], is commonly used as a standard analytical method. ELISA is used not only in human diagnostics but also in the agricultural sector, particularly for the detection of certain plant viruses, fungal phytopathogens, and viral pathogens. Usually, the assay detects proteins (e.g., a viral coat protein) specific to an individual genus or species via polyclonal or monoclonal antibodies [26]. The detection of these proteins is accomplished by complexing antibodies and antigens to produce measurable, usually colored results. The efficiency of the method is often improved by amplifying the primary signal. Although ELISA is the most common means of testing, many other approaches have been described, published, and used in practice.

Other immunoassays, exploiting ready-to-use lateral-flow devices (LFDs), are now available on the market, in which specific antibodies are used for detection and color signaling. Such tests are well-known for their use in the monitoring of human diseases,

e.g., COVID-19, and they are characterized by their ease of use. The results are easy to interpret; thus, they are commonly used for in-field diagnosis. The advantage of LFDs is that they can be used without the need for sophisticated instruments and equipment. These methods are characterized by their speed; they are considered to be rapid, while also being inexpensive and suited to high-throughput diagnostics (Figure 1). On the other hand, other approaches such as DNA-based methods are more sensitive and amenable to multiplexing. LFDs require a priori knowledge of each pathogen. Furthermore, the test is not suited to non-target screening.



**Figure 1.** Detection of *Phytophthora* ssp. in tomato plants infected artificially with *P. infestans* via lateral-flow strips: A1, healthy plant (negative control); A2, negative strip; B2, positive strip; and B1, infected plant.

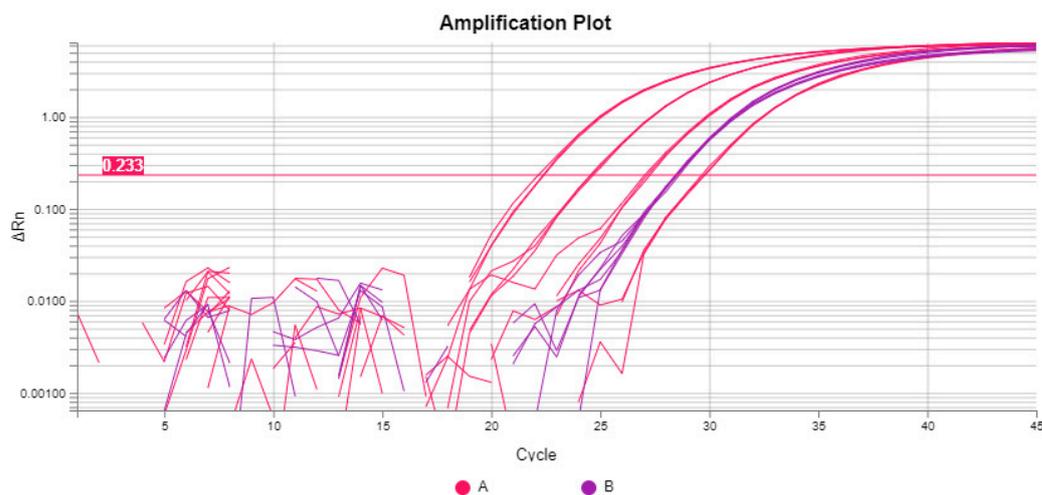
At the same time, a number of DNA/RNA detection approaches have been developed.

#### 4. Polymerase-Chain-Reaction (PCR)-Based Methods for Phytopathogen Detection

Polymerase chain reaction (PCR) is a DNA-based method, as outlined by Mullis and Kaloona [27], that allows for the amplification of selected DNA sequences using a pair of primers, including a short DNA sequence typical of the individual species and individual genotypes surrounding specific DNA stretches. With these primers, along with the enzyme *Taq*, polymerase-multiplied quantities of target sequences can be visualized. PCR is able to capture even a minute amount of the targeted DNA. Theoretically, even a single copy can be captured, but practically speaking, the limit of detection depends on the matrix, and it can be as low as tens of copies of the target. The procedure requires the isolation of DNA/RNA from the analytical target, and the efficiency of the amplification depends on the quality of the extraction process. The important qualities of PCR, as used in diagnostics, are its high specificity, afforded by knowledge of phytopathogen-specific DNA/RNA sequences, and the possibility of designing primers that target selected stretches of DNA [28].

A modification of PCR was introduced a few years ago, when the specificity of PCR was improved as a third-sequence specific primer (probe) was exploited Heid et al. (1996) [29]. In this approach, the probe is fluorescently labelled, and during the synthesis of a new amplicon copy, the fluorescent signal is captured. The approach is composed of real-time PCR (qPCR, if used for target quantification) [30] and digital PCR [31,32]. Real-time PCR may serve as a method for either detection or relative quantification, while digital PCR can be used for the absolute quantification of amplicon copy numbers in the assay. Compared with real-time PCR, digital PCR limits the problems associated with the purity of the extracted DNA through special partitioning. The listed properties of real-time and digital

PCR mean that they are well-suited to a wide range of applications in phytopathogen diagnostics [33]. As PCR-based techniques (Figure 2) are capable of detecting even just a few copies of the target, even a small number of individual virus particles or bacterial or fungal cells can be detected [34]. Thus, these techniques may provide information in the early stages of infection, when the symptoms of infections are barely visible but phytopathogens are already present in the tissue. In addition, multiplex assays, e.g., the detection of several pathogenic species in a single reaction, may be used. Multiplexing reduces the cost and time required to determine the pathogen load [35].



**Figure 2.** Real-time PCR quantification of *Fusarium* fungi in plant tissue: A, calibration standards (red); B, infected sample (violet).

With regard to plant pathogen detection/quantification, we found many protocols for vegetable phytopathogen species, but true validation reports, as required by the Minimal Performance Criteria (MIQE) [36], are not available. The in-lab verification followed by the full validation of published and forthcoming methods is a necessity for researchers to rely on their performance after the provision of comparable results across different laboratories [36–40]. To ensure uniform test performance across laboratories, international standards and guidelines have been issued by the International Organization for Standardization (ISO) that are used by accredited testing laboratories [41]. Thus, PCR-based methods can be used in a harmonized manner.

Since the discovery of the PCR principle, the scope of PCR application has expanded due to the combination with other molecular procedures. One PCR modification, immunocapture–reverse transcription–polymerase chain reaction (IC-RT-PCR) [42], has been suggested as a standard method for the highly sensitive analysis of plants infected with a range of RNA viruses [43,44]. In this method, the preliminary purification of virus particles and viral RNA from the plant material may be bypassed. In addition, in contrast to PCR, all of the ingredients and enzymes in the assay, e.g., temperature, ionic strength, and pH, are able to work under the same conditions. The procedure may, thus, be accomplished in a single step with one tube. IC-RT-PCR was found to be more sensitive than ELISA, being able to distinguish between TMV and ToMV [45,46]. Applications of PCR-based methods for vegetable pathogens are summarized in Tables 1–3.

It is apparent that PCR has undergone a number of modifications [47], but the direct field application of PCR-based methods is limited. For this reason, scientists continue to search for other assays that can be carried out under isothermal conditions.

Farmer-friendly SMART approaches call for small, affordable tools, and some PCR types may be a suitable platform for this purpose. ddPCR (digital droplet PCR) may be a suitable candidate. As the reaction occurs in a greater number of small droplets, Chen et al. [48] suggested using a home-made platform to run the reaction and smartphones to detect the results. As reported, the system could capture several copies of the

target and be used by non-experts. No direct comparison with ddPCR has been published thus far. The design of specific PCR primers requires some knowledge [49,50], which might still be an obstacle for routine farm applications. However, this is a good example of a DIY (do-it-yourself) application.

## 5. Isothermal Amplification

The amplification of nucleic acid is a proven procedure but requires experience, lab equipment, and time. Over the past two decades, interest has increased in the possibility of using isothermal amplification systems as an alternative to the most extensive and long-standing method for amplifying nucleic acids, the polymerase chain reaction. The main advantage of isothermal amplification is that it does not require expensive laboratory equipment for thermal cycling. Thus far, several procedures have been developed [51].

### 5.1. Loop-Mediated Isothermal Amplification (LAMP)

Currently, LAMP (loop-mediated isothermal amplification) of DNA is the most widely used approach implemented under isothermal conditions. It was originally validated in relation to hepatitis B virus (HBV) by Notomi and coworkers [52]. LAMP involves sophisticated primer design and a specific polymerase, and no temperature cycling is needed, thereby relinquishing the need for expensive thermocyclers. Since it was first introduced, this method has become popular, as it is considerably simpler and less demanding than PCR. As the technique is sufficiently robust, LAMP has found application in phytopathogen detection [53–55]. It was soon demonstrated that the technique is applicable to the identification of both DNA and RNA matrices in plants [56]. LAMP products can be detected by the naked eye with the use of neutral red dye, which is another advantage of the method. LAMP diagnostics thus allow for immediate analysis and results without the need for specialized and expensive instrumentation. To date, several options for the visualization of the products have been introduced, as well as possibilities for their combination with smartphone applications [57,58]. Becherer et al. [59] summarized the current knowledge regarding the method's performance parameters (e.g., its robustness, accuracy, precision, reproducibility, and repeatability) under different configurations (e.g., using various types of apparatuses and assays) commonly employed in clinical diagnostics. The authors stated that its diagnostic specificity and sensitivity are well-developed, but they also suggested conducting investigations of its repeatability (intra-assay variance) and reproducibility (inter-assay variance), which were carried out in only 6% of the published cases that they reviewed. This is a serious challenge for researchers developing assays for phytopathogen diagnostics. Moehling et al. [60] highlighted the parameters that should be addressed during assay optimization. Guidelines for validation are offered by the ISO standards [61]. Because of the simplicity of the assay, it can be performed by less-skilled personnel, and this has led to the adoption of the LAMP assay by plant pathologists, opening up possibilities for its use in in-field diagnosis [62]. Currently, several companies offer easy-to-use diagnostic kits, with others in development (see Tables 1–3).

### 5.2. Isothermal Amplification of Nucleic Acid by Recombinant Polymerase—RPA

Another method among the isothermal amplification procedures is recombinase polymerase amplification (RPA) [63]. Here, specific DNA fragments are isothermally amplified using complementary primers resembling those used in PCR for the template DNA, in addition to a set of three enzymes, polymerase, and a DNA-binding protein to enable amplification, instead of the *Taq* polymerase used in PCR. The system allows for the amplification of DNA via synthesis to extend the DNA/RNA strand, directly combining both DNA and RNA [64,65]. This technique has attracted great attention because it is insensitive to *Taq* polymerase inhibitors, and the DNA does not need to be isolated. Previous publications have reported the acceptable limit of detection of the assay [66,67]. Stringent optimization of reaction conditions is highly recommended, as inhibition may occur due to enzyme competition. Piepenburg et al. [64] highlighted the possibility of using a sandwich arrangement

as an apparatus-free assay. In addition, when combined with lateral flow dipsticks (LFD), RPA is reported to be just as fast, accurate, and easy to perform as PCR, thus representing a highly promising approach [68–70]. It is only a matter of time before this procedure is extended to the detection of various phytopathogens. The simultaneous detection of multiple phytopathogenic species is still a problem to be solved, although Bai et al. [71] suggested using RPA in combination with microfluidics in human diagnostics.

### 5.3. SMART Modifications

SMART techniques including DIY (do-it-yourself) devices that do not require well-equipped laboratories are in their infancy. Several examples of DIY technologies have been proposed in medical diagnostics. Bektas et al. [72] introduced a streamline technique called ALERT (Accessible LAMP-Enabled Rapid Test) for COVID-19 detection that is easy to carry out. The procedure can be carried out in one tube but requires PCR primers verified a priori. The COVID-19 assay itself is well-validated. It would be helpful to verify the suitability of the protocol for testing vegetable diseases and to use known LAMP primers. DNA/RNA-specific extraction procedures that complement the ALERT approach are yet to be developed, as vegetable tissues differ in their phenolic compounds and starch contents [73,74]. As farmers are usually not experts in PCR diagnostics, ready-to-use tools need to be provided by commercial companies or at least by scientists.

## 6. CRISPR-Based Techniques

In recent years, new procedures have appeared that can detect and quantify viruses and other phytopathogens in various substrate materials, ranging from human tissues to foodstuffs and environmental components. These are mainly procedures that use not only high-capacity sequencing, but also clustered regularly interspaced short palindromic repeats (CRISPR), and the associated protein, CRISPR/Cas, represents a system that is better-known as a tool used for genome editing [75–77]. As the system is able to easily recognize specific sequences, its usefulness for the detection of specific sequences is apparent. The specific properties of CRISPR/Cas proteins, known as “molecular scissors”, mean that they may be used to bind specified DNA and RNA sequences, making it possible to employ them for diagnostic purposes [78]. CRISPR/Cas-based techniques target both DNA and RNA; thus, they have the advantages of nucleic-acid-based techniques, meaning that high levels of specificity and sensitivity are retained.

The current gold standard for nucleic acid detection—real-time PCR—requires highly qualified personnel and advanced laboratory equipment. The agro-industry is, however, seeking approaches that allow for high-throughput screening and do not require sophisticated reagents or instrumentation. CRISPR/Cas technology, when merged with isothermal technologies, can be used to establish sensitive and cheap detection assays [79], as has been proven in human diagnostics, e.g., for COVID-19 and several other viruses [80]. A CRISPR/Cas-based assay, similar to an isothermal amplification, can be accomplished in less than 30 min at a single temperature, and it can be combined with commercially available fluorescent dye to enable its visualization. These properties render rapid in-field diagnosis easy, as has been demonstrated with plant RNA viruses [81]. In the literature, a simple assay has been described that can be accomplished within an hour and interpreted using simple equipment that is also suitable, in principle, for the diagnosis of viruses in plants. The rapid deployment of assays for the onsite detection of vegetable pathogens is a highly likely future development, and the first examples of such a technology have already published [82,83].

Other studies have shown that it is possible to efficiently combine lateral flow chemistry and CRISPR diagnostics. Based on this idea, there is a possibility of introducing rapid, reliable, specific, and cheap diagnostic kits in the near future [84,85]. In-field applicable and sensitive tools for the vegetable sector may also appear in the near future [86]. Depending on the price of the final product, the utilization of such tools may be widespread. However, Selvam et al. [87] noted that more research on specificity and sensitivity in individual cases

must be performed. Some authors have highlighted that most CRISPR/Cas-based diagnostics are not suited to quantitative detection. However, recently, Wu et al. [88] reported on a new chip system that has been verified for the diagnosis of human pathogens. The assay uses a combination of LAMP, CRISPR/Cas, and digital PCR. The authors claimed that it has superior sensitivity and inhibition tolerance. Such a procedure might be another candidate for vegetable phytopathogen quantification.

## 7. Barcoding

DNA barcoding represents a combination of molecular genetics, DNA sequencing, and bioinformatics approaches for the expeditious, detailed, and automatable cataloging of species using short, conserved gene regions [89]. Barcoding is a procedure in which individual DNA sequences can be used to identify an organism unique to the species level, e.g., a unique identifier. This approach is widely used not only in molecular diagnostics but also in schemes designed to trace goods via barcodes that can be used by customers, e.g., in a supermarket, hence the name “barcoding” (<https://ibol.org/about/dna-barcoding>, accessed on 20 January 2023). These individual DNA sequences possess specific features that are typical of the conserved regions within each species but are highly variable between species. In addition, highly conserved sequences must flank the site to allow for PCR primer design. Several genes have been proven to present such properties, and they are now widely used. Among them, ITSs (ribosomal internal transcribed spacers) are considered to be sufficiently precise. Thus, an ITS was recently designated as the official barcode for fungi [90,91]. Specific sequences, once amplified by PCR, subsequently undergo sequencing. Comparisons of the resulting sequences with the data in existing databases (BOLD, UNITE and NCBI) can then be conducted [92].

Barcoding is used by control laboratories to identify harmful and, in particular, quarantine microorganisms based on the requirements of competent authorities, such as the EPPO, national bodies, or, in the EU, the EU plant health legislation [93].

Currently, the so-called metabarcoding method is employed for the simultaneous identification of multiple targets using multiple genes for barcoding, together with high-throughput sequencing (i.e., next-generation sequencing—NGS) [94]. As observed in nature, multiple phytopathogen infections can occur in a single plant, and their competitiveness, suppression, or synergism may impact on the visible symptoms. The capacity for multiplexing under natural field conditions is therefore highly necessary [95].

Recently, similar to PCR-based methods, guidelines on how to assess the reproducibility of the report were published [96,97]. Precise, validated protocols are required to use the method for control purposes [98].

## 8. Overview of Nucleic-Acid-Based Assays for the Detection of Vegetable Pathogens

In order to map the exploitation of the most frequently used principles reported in the literature, we searched the WOS database for publications over the last 5 years using specific strings of keywords covering purpose (detection, identification, diagnostic), in combination with plant species name and disease names. Published protocols were developed and verified only by single laboratories and thus belong to TRL 4–7, i.e. not directly applicable in wide practice. Through this approach current trends can be assessed.

### *8.1. Application of Different Nucleic-Acid-Based Tools for the Detection of Viral Diseases in Vegetables*

Viruses are invisible entities that damage humans, animals, or plants, including vegetables. They are the most genetically diverse organisms and are rapidly evolving. In general, viruses are characterized by small genomes that encode only a few proteins. Their variability makes it difficult to control them in plants, and DNA-based methods are often used for their detection, as outlined in Table 1.

**Table 1.** List of methods suited to the detection of viral infections of vegetables that have been published in the Web of Science database.

<b>Virus</b>	<b>Method</b>	<b>Species</b>	<b>Reference</b>
<i>Alexivirus</i>	RP PCR	Garlic, onion	[99]
<i>Begomoviruses</i>	RT PCR	Tomato, pepper	[100]
<i>Begomoviruses</i>	LAMP	Chili	[101]
BPMV	Lateral flow +RT CPA	Bean	[102]
BPMV	RT CFA LF	Bean	[102]
CCYV	RT RPA	Cucurbit	[103]
CCYV	RT RPA	Cucumber	[103]
CCYV	RT RPA	Pea	[103]
CGMMV	RTqPCR	Cucumber	[104]
CMV	Immunoassay, DAS ELISA	Cucumber, tomato	[105]
CMV	DAS ELISA	Tomato, pepper	[105]
Multiple	Nested PCR	Multiple	[106]
Multiple	RT PCR	Multiple	[107]
Multiple	NGS	Multiple	[108]
Multiple	PCR, NGS	Multiple	[109]
Multiple	PC, RT PCR	Sweet potato	[110]
Multiple	RT PCR	Garlic, onion	[111]
Multiple	hybridization	Artichoke	[112]
Multiple	DAS-ELISA, IC-RTT PCR	Garlic, onion	[11]
MYMV	CRISPR-Cas (CCI)	Legumes	[86]
OYDV	serological	Garlic, onion	[113]
OYDV	RT PCR+DAS ELISA	Garlic, onion	[113]
OYDV	RT LAMP	Onion	[114]
PLRV, PVY	Isothermal (RT LAMP)	Potato	[115]
PLRV, PVY, PVM, PVA, PVX and PVS.	RT PCR (real-time DiRT-PCR)	Potato	[116]
PMMoV	IC-RT-PCR (TAS-ELISA)	Pepper	[26]
<i>Poleovirus</i>	NGS	Garlic, onion, leek	[117]
PsTDV	RT PCR	Solanum	[118]
PVY	Isothermal RT RPA	Potato	[119]
PVY, PLRV, ToTV and ToCV	NGS	Wild potato	[120]
RNA virus	NGS	Potato	[121]
SPCV	Immunoassay (ELISA)	Potato	[122]
SPLCVs	NGS, Sanger	Sweet potato	[95]
<i>Sweepotvirus</i>	Lateral flow + RT RPA	Sweet potato	[123]
TMV, BWVV2	LAMP	False starwort	[124]
ToBRF	RT PCR	Tomato	[125]
ToBRFV	RT PCR	Tomato	[126]
ToBRFV	ddPCR	Tomato	[127]
ToBRFV	ddPCR	Tomato	[127]

Table 1. Cont.

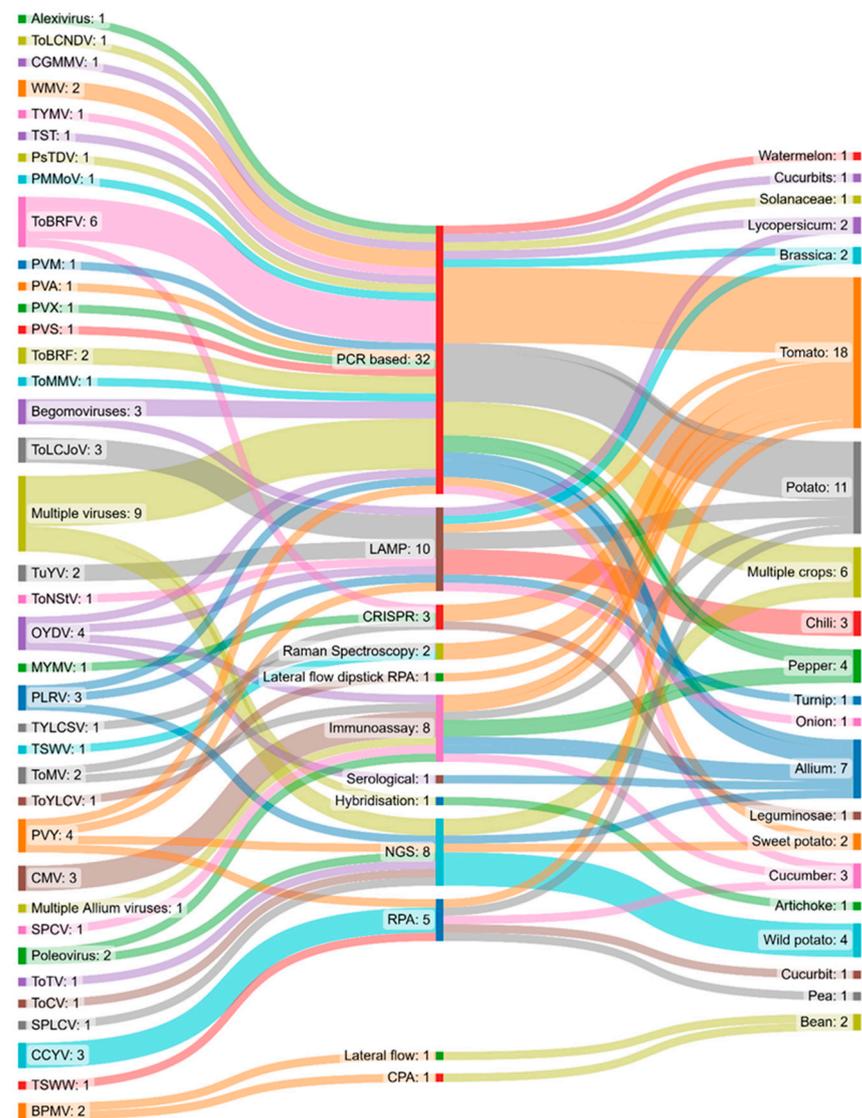
Virus	Method	Species	Reference
ToBRFV	ddPCR	Tomato	[127]
ToBRFV	RT PCR	Tomato	[128]
ToLCJoV	LAMP	Tomato	[101]
ToLCJoV	LAMP	Chili	[101]
ToLCJoV	LAMP	Chili	[101]
ToLCNDV	qPCR	Tomato	[129]
ToMMV, ToBRFV	RT PCRT (duplex)	Tomato	[130]
ToMV	Immunoassay	Tomato	[131]
ToMV, ToBRFV	CRISPR-Cas	Tomato	[132]
ToNStV	RT-LAMP	Tomato	[133]
ToYLCV	Lateral flow dipstick RPA	Tomato	[134]
TST	RT-PCR	Potato	[135]
TSWW	RPA	Tomato	[136]
TuYV	LAMP	Brassica	[137]
TuYV	LAMP isothermal	Turnip	[137]
TYLCSV, TSWV	Raman spectroscopy	Tomato	[138]
TYMV	RT PCR	Brassica	[139]
WMV	RTqPCR	Watermelon, cucurbits	[140]

Figure 3 presents viruses, plant species, and detection methods in a Sankey diagram (generated using the SankeyMATIC online tool, available at <https://sankeymatic.com/build/> accessed on 21 December 2022) and shows interactions between the virus types, methods of detection, and host species. The figure indicates that PCR-based methods have been exploited most frequently during the past five years. The crops for which the greatest numbers of detection methods have been introduced appear to be tomatoes, brassica vegetables, peppers, and chili.

More specifically, it can be observed that the majority of methods are related to the detection of tomato diseases, e.g., ToBRFV or ToLCJoV, as the worldwide tomato production for the year 2022 was reported to be 37.3 million tones [141].

### 8.2. Applications of Different Tools for the Detection of Bacterial Diseases in Vegetables

Bacterial diseases can be visually identified, and their quantification in plants can be achieved by means of colony-counting assays. An infection will have already spread by this stage, and it may be difficult to handle, which is why the prevention of bacterial infections requires early detection. In addition to methods based on barcoding, which are suited to precise identification, PCR is used, including modifications that enable quantification. Several new approaches are currently being tested, including the CRISPR/Cas methodology, which may be used as a tool for genome editing, but also represents a novel, highly precise detection method [142,143]. The table summarizes the methods that have been used over the past 5 years to develop detection assays for bacterial infections published on the WoS. Table 2 summarizes the currently applicable tools for the detection and quantification of various bacterial species.



**Figure 3.** Sankey chart representing the relationships and interconnections between viruses, detection methods, and corresponding crop species.

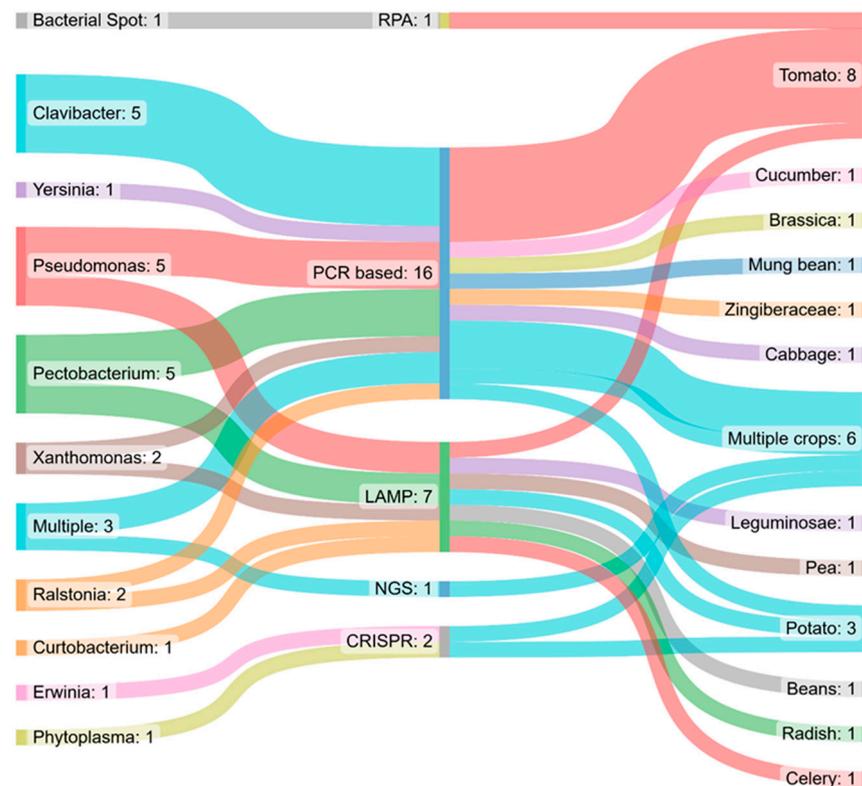
**Table 2.** List of methods suited to the detection of bacterial infections in vegetables that have been published in the Web of Science database.

Bacteria	Method	Species	Reference
Bacterial Spot	RPA	Tomato	[144]
<i>Clavibacter</i>	PCR	Tomato	[145]
<i>Clavibacter</i>	ddPCR	Tomato	[146]
<i>Clavibacter</i>	Multiplex qPCR	Tomato	[147]
<i>Clavibacter</i>	Multiplex qPCR	Tomato	[148]
<i>Clavibacter</i>	Multiplex qPCR	Tomato	[149]
<i>Curtobacterium</i>	LAMP	Legumes	[150]
<i>Erwinia, Acidovorax</i>	CRISPR/Cas	Vegetable	[151]
Multiple	PCR, NGS	Multiple	[109]
Multiple	Bar-coding	Multiple	[152]
<i>Pectobacterium</i>	LAMP	Radish	[153]
<i>Pseudomonas</i>	qPCR	Mung bean	[154]
<i>Pectobacterium</i>	LAMP	Celery	[155]
<i>Pectobacterium</i>	PCR	Cabbage	[156]

**Table 2.** Cont.

Bacteria	Method	Species	Reference
<i>Pectobacterium</i>	Multiplex PCR	Potato	[157]
<i>Pectobacterium</i>	Multiplex qPCR	Multiple	[158]
Phytoplasmas	CRISPR/Cas	Potato	[142]
<i>Pseudomonas</i>	LAMP	Tomato	[159]
<i>Pseudomonas</i>	LAMP	Pea	[160]
<i>Pseudomonas</i>	qPCR	Tomato	[161]
<i>Pseudomonas</i>	Multiplex qPCR	Cucumber	[162]
<i>Ralstonia</i>	LAMP	Potato	[163]
<i>Ralstonia</i>	Multiplex qPCR	Zingiberaceae	[164]
<i>Salmonella, Clavibacter</i>	cultivation	Potato	[165]
<i>Xanthomonas</i>	LAMP	Beans	[166]
<i>Xanthomonas</i>	PCR	Brassica	[167]
<i>Yersinia</i>	ddPCR, qPCR	Multiple	[168]

The plant bacteria detection methods, individual bacterial species, and the corresponding crop species listed in Table 2 are also presented in a Sankey chart (see Figure 4) (produced using the SankeyMATIC online tool, available at <https://sankeymatic.com/build/> accessed on 21 December 2022), allowing for the extraction of significant findings.



**Figure 4.** Sankey chart representing the relationships and interconnections between bacteria, detection methods, and corresponding crop species.

It is apparent that PCR-based tools are still prevalent, but CRISPR/Cas-based methods have also been employed. PCR-based and LAMP techniques are used mainly for tomato crops, and some could be exploited for multiple crops.

The majority of the methods are suited to the detection of *Clavibacter*, *Pseudomonas*, *Pectobacterium*, and *Xanthomonas* species.

### 8.3. Applications of Different Tools for the Detection of Fungal Diseases of Vegetables

Fungi represent a major threat to crop production today and will continue to do so in the future [169]. We retrieved data from the WoS database to identify emerging tools for vegetable fungal pathogen diagnostics described over the last five years. From the overview, one can conclude that research studies have focused on the development of PCR-based assays that are specific to individual fungal phytopathogens. Such assays can be used for the detection of early infections when visual symptoms have not yet been revealed, as in the case of other vegetable pathogens. See Table 3.

**Table 3.** List of methods suited to the identification of fungal infection in vegetables that have been published in the Web of Science database.

Fungus	Method	Species	Reference
<i>Alternaria</i>	Targeted chem.anal.	Tomato	[170]
<i>Atlernaria</i>	Multiplex PCR	Brassica	[171]
<i>Botrytis</i>	Species-specific PCR	Onion	[172]
<i>Botrytis</i>	PCR	Onion	[172]
<i>Botrytis</i>	qPCR	Onion	[173]
Downy mildew	RT PCR	Lettuce	[174]
<i>Erysiphe Palczewski</i>	Visual + smarphone	Robinia	[175]
Foot rot and head rot disease ( <i>S.rolfsii</i> )	ITS PCR seq.	Cabbage	[176]
<i>Fusarium</i>	ITS barcoding	Potato	[177]
<i>Fusarium</i>	Visual/image anal.	Onion	[178]
<i>Fusarium</i>	PCR-RFLP	Pea	[179]
<i>Fusarium</i>	qPCR	Asparagus	[180]
<i>Fusarium</i>	PCR	Tomato	[181]
<i>Fusarium</i>	PCR	Onion	[182]
<i>Fusarium</i>	ITS barcoding	Chili	[183]
<i>Fusarium, Rhizoctonia</i>	Cultivation	Potato	[165]
Multiple	NGS	Tomato	[184]
<i>Hyaloperonospora</i>	Rt PCR	Cucumber	[185]
Late blight	LAMP	Potato, tomato	[186]
Multiple	PCR, NGS	Multiple	[109]
Multiple	e-NOSE	Garlic	[171]
Multiple	Machine vision	Cucumber	[187]
Multiple	ITS RNA seq	Brassica	[187]
Multiple	Image analysis	Brassica	[188]
Multiple	Robotic vision	Bean, pea	[189]
Multiple	ITS RNA seq	Bean	[190]
Multiple	RT PCR	Fabaceae	[191]
Multiple	Machine vision	Cucumber	[185]
Multiple	Multiplex PCR	Cucumber	[192]
Multiple	ITS barcoding	Tomato	[193]
<i>Pectobacterium carotovorum</i>	Sensors	Lettuce	[194]

Table 3. Cont.

Fungus	Method	Species	Reference
<i>Plasmodiophora</i>	Species-specific PCR	Brassica	[195]
<i>Plasmodiophora</i>	PCR, SNPASHOT	Brassica	[196]
Powdery mildew	Spectral data	Cucumber	[197]
Powdery, down mildew	Multiplex qPCR	Cucumber	[198]
<i>Pseudoperonospora</i>	RT PCR	Cucumber	[199]
<i>Rhizoctonia</i>	Multiplex RTPCR	Lettuce	[200]
<i>Rhizoctonia</i>	PCR, RTPCR	Pulse crop	[197]
Rust	IR spectroscopy	Multiple	[201]
<i>Sclerotinia</i>	Spec.specif. PCR	Carrot	[202]
<i>Sclerotinia</i>	RTPCR	Potato	[203]
<i>Sclerotinia</i>	Spec.spec. PCR	Lettuce	[202]
<i>Sclerotinia</i>	Spec.spec. PCR	Lettuce	[204]
<i>Sclerotinia</i>	PCR	Garlic	[205]
<i>Sclerotinia</i>	ITS RNA seq.	Mung bean	[206]
Tomato powdery mildew	RT PCR	Tomato	[207]
<i>Waitea circinata</i>	ITS rDNA seq	<i>Brassica</i> sp.	[208]

In general, the listed PCR-based assays are highly sensitive. Based on the literature reviewed in the current study, the fungi, detection methods, and the corresponding crop species presented in Table 3 are also presented in a Sankey chart shown in Figure 5 (produced using the SankeyMATIC online tool, available at <https://sankeymatic.com/build/> accessed on 21 December 2022), allowing for the extraction of significant findings.

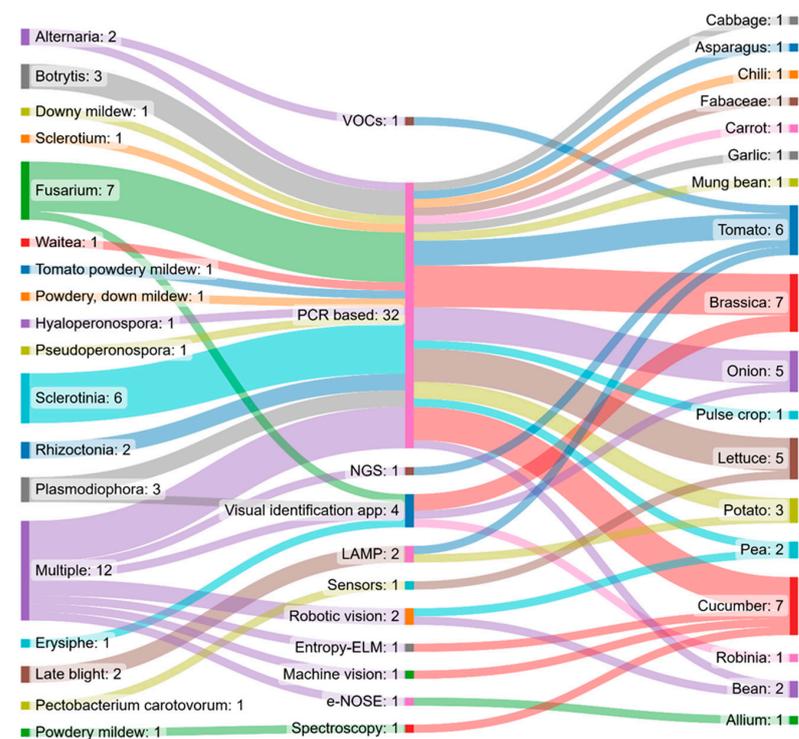


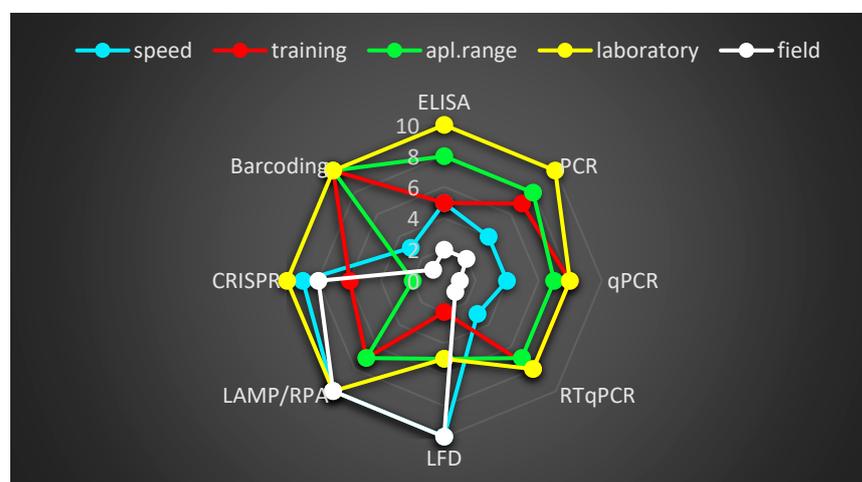
Figure 5. Sankey chart representing the relations and interconnections between fungi, detection methods, and corresponding crop species.

It is apparent that most of the published methods refer to the detection of *Fusarium* and *Sclerotinia* species. The most widely used detection techniques for fungi are nucleic-acid-based techniques, and the visual identification applications mainly concern tomato, cucumber, and brassica crops.

#### 8.4. Commercially Available IPM Tools/Solutions

Practical applications based on research have been developed, proofs-of-concept have been verified, and several tools have reached the highest level of technological readiness, TRL 9 [209], which means that these tools are available on the market. Descriptions and assessments of these methods are available at <https://platform.smartprotect-h2020.eu/en> (accessed on 2 January 2023), a database developed by the SmartProtect consortium.

Generally, the attributes of these techniques are assessed according to their applicability, e.g., the range of pathogens they can identify; their requirements for specific equipment; the education and training necessary for the operators; their speed in making determinations; and their availability in individual markets. Figure 6 shows the positive attributes of the principal, most widely used methods.



**Figure 6.** Comparative analysis of different techniques used for plant diagnosis based on their basic properties (speed, demand for trained personnel, application, level of technological readiness (TRL8 or TRL9), availability of the test in laboratories, and possibility of application in the field).

It is clear that test strips are suitable for quick use on farms and in greenhouses; it follows that their use for accurate determination is even easier in laboratories.

## 9. Biosensors

Biosensors are among the best approaches for obtaining easy and cheap diagnostics. In principle, biosensors are devices that, in our case, measure biological reactions, giving rise to a signal equivalent to the amount of target. Biosensors rely on a combination of biorecognition molecules and transducers converting one type of variation into another, e.g., an electric signal and a visualization system, making them easy to use [210]. They were first used in on-site diagnostics to identify human diseases [211–213]. The current techniques for producing biosensors aim to employ new materials, being manufactured using nanotechnologies and possessing higher biological affinity, to develop new sensors capable of disease surveillance on different scales. The concept has been used for detection of human pathogens on marketed vegetables [214–216]. To date, research has mainly focused on human pathogens that contaminate vegetable production, such as *Salmonella* or *Listeria* [215,217,218]. Few reports describe the use of biosensors for vegetable phytopathogen detection [215,219,220]. The use of biosensors for the precise detection of vegetable phytopathogens needs to be evaluated in practice. Hopefully, they will find

application in the detection and quantification of pathogens that contaminate vegetables in the near future [221].

## 10. E-Senses

Other approaches do not use species-/pathotype-specific proteins, such as ELISA, or DNA/RNA, such as PCR or sequencing, as a target may be aimed towards metabolites (eNose), visual patterns such as color changes, structural changes (eEyes), or differences in soluble materials (eTongue), resembling the functions of the human senses. Numerous papers have been published describing the basic principles of these assays and showing the current state of the art [222–227].

Among the e-Senses, eNose is an approach that allows for the detection of volatiles released by organisms and senses volatiles using miniaturized gas chromatography instruments [228]. In the case of eNose, focus is placed on specific metabolites produced by the pathogen itself and by infected plants.

It is known that volatiles produced by an organism undergo changes related to their physiological state and developmental stage. Hundreds of reports are available on compounds captured upon plant infection [229,230] by various types of chromatography and mass spectrometry [231,232]. Thus, with libraries of volatile mixtures similar to those continuously supplemented for regular metabolomics analysis [233,234] produced upon infection, simple tools might be available for farmers in the future to conduct large-scale analyses of crops, including vegetables. Some authors [235,236] have reported on the practical utilization of eNose for palm tree disease detection. Borowik et al. [237] reported on the successful utilization of eNose for the detection of *Phytophthora* in *Quercus* acorn and later, with an improved chamber, *Ciboria batschiana* [238]. The same group [237] introduced a low-cost detection procedure for phytopathogenic fungi. Although several examples of eNose utilization have been described in the literature, only a few have undertaken the detection of plant pathogens, and applications with vegetables have seldom been carried out. Only one example was found to be commercially available (TRL9; for further details, see the SMARTPROTECT platform database). Additionally, the authors of [239] noted that eNoses may find widespread application for plant diseases diagnostics, as well as other purposes, but more research and testing are required before incorporating this efficient tool into agriculture practice [239]. To spread the techniques used for various vegetable species cultivated across the world and the detection of their pathogens, tremendous effort will be required to combine the technical basis of e-Noses and sensing of specific mixtures of compounds. Further knowledge and verification are needed. Nevertheless, Mohammad-Razdari et al. [240] predicted their potential for farm management. Direct comparisons with conventional laboratory methods will be essential for routine application in practice.

## 11. Outlook

The timely, correct, and accurate diagnosis of diseases is the fundamental basis for the successful cultivation of agricultural crops, including vegetables. Along with early warning systems, diagnostic tools are essential for disease management and the production of high-quality and high-yielding vegetables. Currently, the diagnosis of plant diseases is based on approaches developed by other sectors, e.g., medicine, and generally, they are first applied to major crops, followed by later extension to vegetables. Commercially, specific procedures such as ELISA, PCR, real-time PCR, and isothermal amplification are available, but these require a priori knowledge of the target molecules used for each pathogen, and they differ in their level of sensitivity (detection limit). Other tests have been described in the scientific literature, but few of these that are applicable to vegetables have been adequately tested in regard to their analytical parameters, in contrast to their use in human medicine. It can be assumed that users will demand multiple assays, enabling the multiplex detection of a number of pathogens simultaneously, in addition to next-generation sequencing (NGS) and barcoding. Thus far, the difficulties lie in the high cost of such methods and in interpreting the results. With decreases in the cost of the equipment and services and the development

of simpler interpretation platforms, this type of technology has the potential to be extended to use on vegetables. A second advantageous route lies in the development of simple diagnostics in the field. Currently, isothermal amplification is advantageous due to its ease of use and quick results. However, the use of CRISPR/Cas for diagnostics, in combination with suitable carriers (test strips), appears to be a highly promising method, according to publications from 2021–2022. This is especially true considering the high accuracy of the system and the possibility of signal amplification. Overall, it is clear that the greatest challenge for phytopathogen diagnostics is the production of affordably priced assays that carry the possibility of multiple detections through the analysis of a single sample.

The evaluation of commercially available solutions shows that there is a wealth of lateral flow test solutions covering a wide range of pathogens and crops, while simultaneously forgoing requirements for specialized equipment or training. The ELISA, LAMP, and PCR/qPCR methods are more accurate but require specialized equipment, more time, and trained users to perform the analyses. Thus, lateral flow tests constitute very useful preliminary tests, being performed prior to laboratory analyses, and they can offer an early indication of viral, fungal, and bacterial infections.

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