Diagnosis and Detection of Soil-Borne Fungal Phytopathogens in Major Crops

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Abstract

Phytopathogenic soil-borne fungal species can inflict huge economic disturbances in the global agricultural sector. Soil-borne diseases, incited by fungal pathogens, e.g. root rot, stem rot, crown rot, damping-off, blights, vascular wilts, etc., inflict significant economic losses in agricultural and horticultural crops' yields and quality, globally. To achieve effective disease control, precise and quick detection or identification of plant infecting fungi is required. For accurate plant disease diagnosis, DNA-based approaches have become widespread. Recent breakthroughs in the field of fungal detection and differentiation; various polymerase chain reaction (PCR) assays such as nested, multiplex, quantitative, bio, and magnetic-capture hybridisation PCR techniques; post and isothermal amplification methods; DNA and RNA-based probe development; and next-generation sequencing have resulted in novel molecular diagnostic tools. Symptomatic and asymptomatic diseases caused by culturable and non-culturable fungal pathogens can be detected using these molecular-based detection approaches in both single-infection and co-infection conditions. Plant disease diagnostics require molecular techniques that are more reliable, quicker, and

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easier to use than traditional procedures. The present chapter highlights molecular diagnostic tools that have come a long way including rapid developments in recent past. However, it requires further firming up before becoming integral part of efficient plant disease management.

Keywords

Agricultural crops · Disease diagnosis · Molecular detection · Phytopathogens · Soil-borne fungi

4.1 Introduction

Plant diseases are very important as they have posed historical impacts on the human civilisation globally, and even in recent era, they are capable enough to cause great economic losses and can raise concerns for food safety around the world (Kumar and Gupta 2020). To the convenience of the study, plant pathologists have classified plant diseases into three major distinct groups: (a) seed-borne diseases, (b) soil-borne diseases, and (c) air-borne diseases. There are no clear dividing lines between these three groups, and a disease may use one or more mechanisms to spread or survive. For instance, the loose smut disease of wheat incited by *Ustilago segetum* sp. *tritici* is an entirely seed-borne and seed-transmitted disease (Kumar et al. 2020). The dormant mycelium of the pathogen remains deep seated in the seed embryo. The mycelium becomes activated and grows alongside the host plant with no visible symptoms, when these contaminated seeds are sown in the field. The pathogen expresses itself after ear emergence only, and instead of healthy spikelets, smutted ones with mass of millions of teliospores arise. These teliospores are blown away by the wind after sometime, allowing them to infect new plants (Gupta and Kumar 2020b). As a result, we can observe that, although being seed-borne, the ailment needs the support of air to complete its life cycle. The nature of the disease is determined by the primary commencement of disease transmission. There are also some diseases where the primary source of infection might come from a variety of sources. The bakanae, or paddy foot rot disease, is an example. Although the pathogen of this disease, *Fusarium moniliforme*, is thought to be primarily seed-borne, inoculum of *Fusarium moniliforme* present in the soil is capable of infecting rice plants with bakanae disease (Gupta et al. 2015; Gupta and Kumar 2020a). Similarly, the Karnal bunt of wheat relies on all three pathways for survival and spread: soil-borne, air-borne, and seed-borne (seed co-contaminant) (Kumar and Gupta 2020; Kumar et al. 2020).

Soil-borne diseases caused by diverse soil-dwelling microbes are among the most challenging threats to agriculture production worldwide. These diseases are very difficult to manage due to the complexity in delivery of the pesticides efficiently at target pathogen's site in the soil. Moreover, the symptoms produced on aerial and underground parts are very similar in case of many soil-borne diseases. Hence, timely and efficient detection of these diseases and their inciting pathogens is

prerequisite for effective disease management (Kumar et al. 2008). The current chapter discusses recent advances in the development and utilisation of molecular approaches for identification of established and emerging soil-borne plant pathogenic fungi.

4.2 Soil-Borne Plant Pathogens Produce a Variety of Symptoms

4.2.1 Rotten Roots

A wide array of fungus and associated organisms cause soil-borne diseases. *Pythium* and *Phytophthora*, *Rhizoctonia*, *Cylindrocladium*, and *Armillaria* are the most common genera that cause root rots. The symptoms of these diseases are the breakdown of the actual root system; certain pathogens are exclusive to the juvenile roots, while others can affect the older root system. Wilting, leaf death and fall, branch and limb death, and, in severe situations, the death of the entire plant are all apparent indications. The following are some examples of these disorders:

4.2.2 *Rhizoctonia* Root Rot Disease

The words "damping-off", "wire stem", "head rot", and "crown rot" all refer to the same issue. The fungus only infects the outer cortical tissues of older seedlings, causing a lesion that is elongated and tans to reddish-brown in colour. The zone may widen and lengthen until it encircles the stem; when this happens, the plant will die.

4.2.3 Stem, Collar, and Head Rots

These diseases are caused by a variety of pathogens, including *Phytophthora*, *Sclerotium*, *Rhizoctonia*, *Sclerotinia*, *Fusarium*, and *Aspergillus niger*. The most evident sign of these diseases is the degeneration of the stem at ground level. Wilting symptoms, leaf death, and plant death are all common consequences of this degradation. The following are some examples of these disorders. *Phytophthora* spp. can cause various diseases including pineapple heart rot, potato and tomato blight, and numerous fruit rots in these conditions. In damp, warm conditions, *Rhizoctonia* spp. can cause maize leaf blight and cabbage head rot.

4.2.4 Wilts

Fusarium oxysporum and *Verticillium* spp. are the two most frequent fungi that cause these infections. This disease results in internal necrosis of the vascular tissue

in the plant's stem and wilting of the foliage. Similar to how some bacterial species can lead to the same.

4.2.5 Blights on Seedlings and Damping-Off Diseases

Seedling diseases are known by a variety of names, including seedling blight and damping-off. *Pythium*, *Phytophthora*, *Rhizoctonia*, *Sclerotium rolfsii*, and *Fusarium* spp. are the most frequent fungus that kill seedlings. Several fungi can infect seedlings during the germination, pre-emergence, or post-emergence stages of seedling establishment. *Pythium*, *Rhizoctonia*, and *Sclerotium rolfsii* are frequently linked to seedling death in vegetables like beans, tomatoes, cucurbits, and other cruciferous plants.

4.2.6 *Pythium* Damping-Off Disease

Pythium debaryanum, *Pythium ultimum*, *Pythium aphanidermatum*, and *Pythium graminicola* are the most common species found. The disease frequently manifests itself in a nearly circular pattern. This is due to fungi's proclivity for rapidly spreading from their source, which is one of the field markers used to distinguish illnesses from other causes that produce similar symptoms.

4.2.7 Damping-Off *Phytophthora*

The *Pythiaceae* family includes *Phytophthora* species, which are classed as *Oomycetes*. Low stem rot, or damping, is caused by *P. cactorum*, *P. fragariae*, *P. palmivora*, and *P. syringae* on vegetables, forest trees, and ornamentals. *Phytophthora* is more active than *Pythium* in warmer soil temperatures (15–23 °C), although it still thrives in a cold environment. Flooding and hot temperatures are the order of the day. At first, the injured tissue develops a mushy, watery brown rot. The plant parts that have been damaged may dry out in a few days.

4.2.8 Major Soil-Borne Disease Caused by Fungal Pathogens

The agents that induce soil-borne diseases make up a diverse group. Fungi, which are multicellular microorganisms, are considered as major soil-borne pathogens causing diseases in cereals, pulse, oilseed, fruit, vegetables, crops, etc. Some important soil-borne diseases of cultivated crops are mentioned in Table 4.1 and Fig. 4.1 along with some pathogenic fungi in Fig. 4.2.

Table 4.1 Some important soil-borne fungal diseases and their phytopathogens in various agricultural crops

S. no.	Crop	Disease name	Fungal pathogen	Reference
1.	*Alliums*	Damping-off	*Pythium* spp., *Rhizoctonia* spp.	Sharma et al. (2022)
		Basal rot	*Fusarium oxysporum* f. sp. *cepae*	Le et al. (2021)
		Pink rot	*Phoma terrestris*	Mishra et al. (2012)
		White rot	*Sclerotium cepivorum*	Zewide et al. (2007)
2.	Banana	Panama wilt	*Fusarium oxysporum* f. sp. *cubense*	Aguilar-Hawod (2020)
3.	Bean	Ashy stem blight	*Macrophomina phaseolina*	Díaz-Díaz et al. (2022)
4.	Brinjal	Collar rot	*Sclerotium rolfsii*	Jadon (2009)
5.	Carrot	Cavity spot	*Pythium violae*	Lyons and White (2008)
		Cottony rot	*Sclerotinia sclerotiorum*	Kora et al. (2003)
		Crown rot	*Rhizoctonia solani*	Marcou et al. (2021)
		Southern blight	*Sclerotium rolfsii*	Rubayet et al. (2020)
		Phytophthora root rot	*Phytophthora* spp.	Williamson-Benavides and Dhingra (2021)
		Root die back	*Pythium* spp.	Kalu et al. (1976)
6.	Celery	Crater spot	*Rhizoctonia solani*	Houston and Kendrick (1949)
		Fusarium yellows	*Fusarium oxysporum* f. sp. *apii*	Epstein et al. (2017)
		Pink rot	*Sclerotinia sclerotiorum*	Bolton et al. (2005)
7.	Chickpea	Collar rot	*Sclerotium rolfsii*	Javaid and Khan (2016)
8.	Chilli	Foot rot	*Sclerotium rolfsii*	Sultana (2012)
		Southern blight	*Sclerotium rolfsii*	Sharf et al. (2021)
9.	Cole crops	Bottom rot and wire stem	*Rhizoctonia solani*	Keinath (2019)
		Club root	*Plasmodiophora brassicae*	Yu et al. (2022)
		Fusarium yellows	*Fusarium oxysporum* f. sp. *conglutinans*	Yu et al. (2020)
		Root rot	*Phytophthora megasperma*	Williamson-Benavides and Dhingra (2021)

(continued)

Table 4.1 (continued)

S. no.	Crop	Disease name	Fungal pathogen	Reference
		Verticillium wilt	*Verticillium dahliae*	Kowalska (2021)
		White mould	*Sclerotinia sclerotiorum*, *S. minor*	Faraghati et al. (2022)
		White rust	*Albugo candida*	Asif et al. (2017)
10.	Cucumber, melons, squash	Charcoal rot	*Macrophomina phaseolina*	Marquez et al. (2021)
		Damping-off	*Pythium* spp., *Rhizoctonia solani*	Lamichhane et al. (2017)
		Fusarium wilt	*F. oxysporum* f. sp. *melonis* (muskmelon); *F. oxysporum* f. sp. *niveum* (watermelon); *F. oxysporum* f. sp. *cucumerinum* (cucumber)	Egel and Martyn (2007)
11.	Finger millet	Foot rot	*Sclerotium rolfsii*	Manu et al. (2012)
12.	Groundnut	Stem rot	*Sclerotium rolfsii*	Jacob et al. (2018)
13.	Guava	Wilt	*Fusarium oxysporum* f. sp. *psidii*	Srivastava et al. (2011), Singh et al. (2021)
14.	Indian mustard	Sclerotinia rot	*Sclerotinia sclerotiorum*	Singh et al. (2020)
15.	Lentil	Foot/root rot	*Sclerotium rolfsii*	Khalequzzaman (2016)
16.	Lettuce	Bottom rot	*Rhizoctonia solani*	Wallon et al. (2021)
		Lettuce drop disease	*Sclerotinia sclerotiorum* and *S. minor*	Mihajlović et al. (2022)
		Wilt	*Fusarium oxysporum* f. sp. *lactucum*	Egel and Martyn (2007)
17.	Maize	Stalk rot	*Fusarium moniliforme*	Jiskani et al. (2021)
		Stem rot	*Sclerotium rolfsii*	Soytong (1991)
18.	Pea	Damping-off	*Pythium* spp., *Rhizoctonia solani*	Lamichhane et al. (2017)
		Fusarium root rot	*F. solani* f. sp. *phaseoli*	Wu et al. (2022)
19.	Pepper	Damping-off	*Pythium* spp., *Phytophthora* spp., *Rhizoctonia solani*	Lamichhane et al. (2017)
		Root rot	*Phytophthora capsici*	Lozada et al. (2021)
		Verticillium wilt	*Verticillium dahliae*	Kowalska (2021)
20.	Potato	Black dot	*Colletotrichum atramentarium*	Lees and Hilton (2003)

(continued)

Table 4.1 (continued)

S. no.	Crop	Disease name	Fungal pathogen	Reference
		Black scurf	*Rhizoctonia solani*	Tjimune et al. (2021)
		Charcoal rot	*Macrophomina phaseolina*	Marquez et al. (2021)
		Fusarium dry rot	*Fusarium sambucinum*	Erper et al. (2022)
		Leak	*Pythium* spp.	Çakır et al. (2020)
		Pink rot	*Phytophthora erythroseptica*	Çakır et al. (2020)
		Powdery scab	*Spongospora subterranea*	Tsror et al. (2020)
		Silver scurf	*Helminthosporium solani*	Tiwari et al. (2022)
		Verticillium wilt	*Verticillium dahliae*	Kowalska (2021)
		White mould	*Sclerotinia sclerotiorum*	Ojaghian (2018)
21.	Rice	Bakanae	*Fusarium fujikuroi*	Jiang et al. (2021)
		Sheath blight	*Rhizoctonia solani*	Senapati et al. (2022)
		Stem rot	*Sclerotium oryzae*	Ghosh et al. (2020)
22.	Soybean	Collar/foot/ root rot	*Sclerotium rolfsii*	Borah and Gogoi (2020)
		Charcoal rot	*Macrophomina phaseolina*	Bradley and Río (2003)
23.	Spinach	Damping-off	*Fusarium oxysporum*, *Pythium* spp., *Rhizoctonia solani*	Sharma et al. (2022)
24.	Sugar beet	Collar/root rot	*Sclerotium rolfsii*	Rasu et al. (2013)
25.	Sugarcane	Pokkahboeng	*Fusarium moniliforme*	Srivastava et al. (2020b)
26.	Sunflower	Charcoal rot	*Macrophomina phaseolina*	Weems et al. (2011)
		Collar/root rot	*Sclerotium rolfsii*	Rasu et al. (2013)
27.	Strawberry	Crown rot	*Macrophomina phaseolina*	Mertely et al. (2005)
28.	Tomato	Wilt	*Fusarium oxysporum* f. sp. *lycopersici*	Katyayani et al. (2019), Manda et al. (2021)
		Damping-off	*Pythium* spp., *Phytophthora* spp., *Rhizoctonia solani*, *Sclerotium rolfsii*	Sharma et al. (2022)

(continued)

Table 4.1 (continued)

S. no.	Crop	Disease name	Fungal pathogen	Reference
		Foot rot	*Fusarium solani*	Ribeiro et al. (2022)
		Verticillium wilt	*Verticillium dahliae*	Mazzotta et al. (2022)
29.	Wheat	Root rot	*Sclerotium rolfsii*	Elad et al. (1980)
		Foot rot	*Rhizoctonia solani*	Ophel-Keller et al. (2008)
		Dwarf bunt	*Tilletia controversa*	Yuan et al. (2009)
		Take-all disease	*Gaeumannomyces graminis* var. *tritici*	Ophel-Keller et al. (2008)
		crown rot	*Fusarium pseudograminearum* and *F. culmorum*	Ophel-Keller et al. (2008)
		Root rot, crown rot, and spot blotch	*Bipolaris sorokiniana*	Ophel-Keller et al. (2008)

4.3 Detection Methods of Soil-Borne Plant Pathogenic Fungal Species

4.3.1 Traditional Methods

Identifying disease indications, direct isolation in artificial conditions, and laboratory identification by morphological or biochemical assays have all been used in the past. These methods required an experienced and competent laboratory staff to perform them since they could result in problems with identification, erroneous findings interpretation, improper disease diagnosis, and, ultimately, incorrect disease therapy (Atkins and Clark 2004; Martinelli et al. 2015). Furthermore, these methods are time-consuming, non-quantitative, and prone to contamination and mistakes and result in major delays in plant treatment. Although molecular technologies are becoming more readily available, conventional procedures are still frequently employed and are the mainstay of plant pathologists.

Traditional approaches for identifying soil-borne infections, such as baiting and the use of selective media, such as Botrytis Selective Media (BSM) for *Botrytis cinerea*, because they are inexpensive and not technically demanding, are used extensively (Horner and Wilcox 1995, 1996; Pryor et al. 1998; Edwards and Seddon 2001). They are, however, often time-consuming, error-prone, and occasionally erroneous, and they necessitate a thorough understanding of classical taxonomy as well as a high level of competence for interpretation and analysis. They are not well suited to large-scale sample analysis or rapid diagnosis, and producers must rely on specialised diagnostic facilities. Other drawbacks include the inability to precisely

Fig. 4.1 Some major diseases of agricultural crops having soil-borne phase in their disease cycle; false smut (**a**), bakanae (**b**), sheath blight (**c**) of rice; flag smut (**d**), Karnal bunt (**e**), spot blotch (**f**) of wheat; spot blotch of barley (**g**); late blight of potato (**h**), late blight of tomato (**i**), *Sclerotinia* stem rot of mustard (**j**); smut (**k**), ergot (**l**), and downy mildew or green ear disease (**m**) of pearl millet

identify infections and the difficulties of culturing some species in vitro (Ghosh et al. 2015; Sharma et al. 2015). Using a combination of traditional pathogen knowledge and molecular detection technologies, these constraints could be overcome with greater precision and reliability (Ghosh et al. 2019). Therefore, the focus of this chapter is on emerging molecular approaches that are increasingly being employed for the detection and identification of diseases which spread through soil.

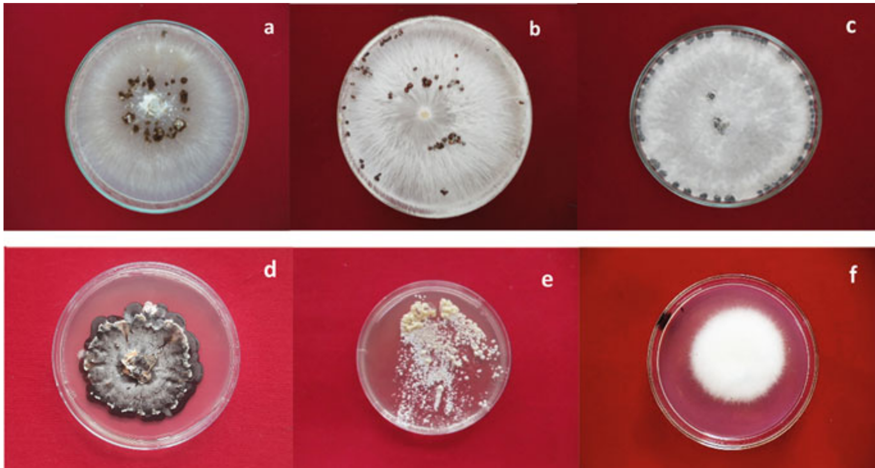

Fig. 4.2 Some important soil-borne phytopathogens; *Rhizoctonia solani* (**a**), *Sclerotium rolfsii* (**b**), *Sclerotinia sclerotiorum* (**c**), *Bipolaris sorokiniana* (**d**), *Tilletia indica* (**e**), and *Fusarium moniliforme* (**f**)

4.3.2 Recent Detection Techniques for Soil-Borne Fungal Phytopathogens

Since the traceable early history of detection and diagnosis of plant diseases, the methodologies employed for the detection and identification of the pathogens were knowingly or unknowingly being judged on the certain criteria such as ease of performance, reliability, scientific reasoning, etc., before validation and widespread adoption of these techniques (Srivastava et al. 2020a). Ball and Reeves (1991) devised six main requirements for selection of detection techniques in case of seed-borne pathogens, which may be applicable to other kinds of the phytopathogens. The technology to be employed for detection of phytopathogens must have to fulfil six main requirements (Ball and Reeves 1991) with some modification in case of soil-borne fungal pathogens as given below:

(a) **Specificity**—a target organism's ability to be distinguished from others found on tested samples.
(b) **Sensitivity**—the capacity to find organisms in samples with low occurrence.
(c) **Speed**—little time is needed, allowing for quick action against the target pathogen(s).
(d) **Simplicity**—reduction of several testing phases to lower error and allow testing by a team that isn't always extremely competent.
(e) **Cost-effectiveness**—costs should determine acceptance to the test.
(f) **Reliability**—regardless of who conducts the test, techniques must be sufficiently reliable to produce reproducible results both within and across samples of the same stock.

The molecular techniques employed for detection of soil-borne fungal phytopathogens includes several techniques, viz. conventional PCR, real-time PCR, end-point PCR, bio-PCR, nested-PCR, multiplex-PCR, RPA, LAMP, RCA, NASBA, FISH, etc. For the species-specific detection of fungal phytopathogens, technologies with a high level of sensitivity and specificity are employed. With the help of these procedures, diseases of different crops can be found using incredibly little samples or tissues. Due to their specificity, sensitivity, speed, simplicity, and reliability, molecular detection methods have recently taken the lead in the detection, identification, and quantification of soil-borne fungal pathogens. To some extent, these methods are also cost-effective, especially when samples need to be diagnosed in bulk. Therefore, we can say in recent times use of molecular techniques must be preferred over any other available conventional techniques for better understanding, interpretation, and accuracy. Some important techniques are as follows:

4.3.2.1 PCR-Based Approaches

Conventional PCR

PCR is a strong technology for amplification of DNA sequences exponentially. A PCR process requires a pair of primers that are complementary to the sequence of interest. The DNA polymerase extends the primers. The amplicons, or copies created after the extension, are re-amplified with the same primers, resulting in exponential amplification of the DNA molecules. The amplified PCR products are next analysed using gel electrophoresis, which makes conventional PCR time-consuming because the reaction must end before the post-PCR analysis can begin. Real-time PCR tackles this problem by detecting the amount of PCR product while the reaction is still in the exponential phase, thanks to its ability to quantify PCR amplicons as they accumulate in a "Real Time Detection" mode (qPCR).

Real-Time PCR

Real-time polymerase chain reaction (real-time PCR), commonly referred to as quantitative polymerase chain reaction (qPCR), is a molecular biology laboratory technique. Instead of waiting until the end, like in conventional PCR, it monitors the amplification of a particular DNA molecule during the PCR (in real time). This method is an upgraded version of traditional PCR in which the DNA may be quantified while the amplification is taking place (Mackay 2004). The proportional number of copies of the target DNA and RNA sequences can be calculated by extrapolating the Ct (cycle threshold) value of the fungal samples using sequence-specific primers (Balodi et al. 2017). The use of fluorescent dyes like SYBR Green I or sequence-specific fluorescence-labelled probes like the TaqMan probe has allowed for monitoring of reactions during amplification steps (Badali and Nabili 2012). Fluorescent signal is produced when the fluorescent dye intercalates with DNA. After each cycle of amplification, this signal grows as the amount of targeted DNA grows (McCartney et al. 2003; Alemu 2014). The fluorescent dye is less expensive as a monitoring agent; however, it has limitations due to its non-specific character. Intercalating dye binding to all existing DNA might, in fact, provide

erroneous findings in the form of primer dimer. Then, because of their great specificity, fluorogenic probes became popular (Atkins and Clark 2004; Bu et al. 2005). Two types of fluorescent dyes are attached to these probes: one is a reporter dye that attaches to the 5′ end, and the other is a quencher dye that attaches to the 3′ end. The emission of fluorescence is prevented by the close proximity of the reporter and the quenching dye. Taq polymerase's exonuclease activity causes the reporter dye to detach from the quenching dye and fluoresce (Dasmahapatra and Mallet 2006). The disease-causing fungus *Aspergillus versicolor*, *Cladosporium cladosporioides*, *Stachybotrys chartarum*, and *Alternaria alternata* have all been identified and quantified using qPCR (Black 2009).

The sensitivity of real-time PCR appears to be higher than that of conventional PCR. With real-time PCR, amplification of *Rhizoctonia solani* target DNA isolated from soil was achieved at 900 bp, but not with traditional PCR (Lees et al. 2002). Similarly, a TaqMan-based PCR yielded the same level of sensitivity for specific identification of *Helminthosporium solani* in soil and tubers (Cullen et al. 2001). A further boost in sensitivity can be reached by combining two consecutive amplifications with conventional (first amplification) and labelled primers (second amplification) without sacrificing the benefits of real-time PCR. *Rosellinia necatrix* (Schena et al. 2002; Schena and Ippolito 2003), *Verticillium dahliae* (Nigro et al. 2002), *Phytophthora nicotianae*, and *P. citrophthora* (Ippolito et al. 2000) were detected using this method (nested Scorpion-PCR) on different substrates (soils, roots, bark, and/or woody tissues) that are naturally infected. Nested Scorpion-PCR produced higher levels of sensitivity and took substantially less time than traditional detection procedures (Schena et al. 2004). A real-time PCR-based marker for the detection of *Tilletia indica* teliospores in soil was recently created (Gurjar et al. 2017).

Cryphonectria parasitica is a hypervirulent and emerging fungal plant pathogen that produces blight, deadly cankers on bark, dieback, and wilting in chestnut trees, *Castanea dentata*, and *C. sativa* (Murolo et al. 2018; Jain et al. 2019). With the aid of rDNA ITS sequences, qPCR was able to identify *C. parasitica* with a sensitivity of 2 fg of genomic DNA, which is equal to a single spore of the disease (Chandelier et al. 2019). *Ramularia collo-cygni*, a newly discovered fungal pathogen, causes little dark patches on leaves, sheaths, and awns, making it tough to analyse the disease by using traditional methods (Havis et al. 2015). The first report on the molecular identification of *Ramularia collo-cygni* in barley seed was developed and submitted using a qPCR assay (Havis et al. 2014). Another novel fungal pathogen identified by qPCR is a fast-growing and aggressive British *Verticillium longisporum* (Depotter et al. 2017). The fungi that produce *Phomopsis* stem canker in sunflowers, *Diaporthe helianthi* and *Diaporthe gulyae*, were discovered and quantified using qPCR. The assay was used to successfully screen these causal compounds from the same genus (Elverson et al. 2020). *Pyrenophora tritici-repentis* and *Parastagonospora nodorum* co-infect wheat and have similar physiognomies, making traditional disease identification difficult. To execute a duplex qPCR test, two dual-labelled probes with unique fluorogenic reporters were custom built (permitting parallel but independent amplification of DNA sequences from

P. tritici-repentis and *Pa. nodorum*), and the results were precise and suitable for simultaneous variation, as well as for high-throughput screening of several diseases (Abdullah et al. 2018). This method is rapid and accurate (Sikdar et al. 2014), and it can provide precise pathogen load information (Garrido et al. 2009), as well as high-throughput detection of target DNA in biological domains (Schena et al. 2013). Additionally, the TaqMan probe adds another degree of specificity (Shuey et al. 2014). Nevertheless, qPCR necessitates the use of a specialised equipment, which can be costly both in terms of the device and the probe (Abdullah et al. 2018).

End-Point PCR

The use of PCR revolutionised the reliable detection of many plant pathogens, including fungi a prerequisite for disease control (Ma and Michailides 2007). A fragment of DNA template is exponentially amplified in this in vitro process (Caetano-Anolles 2013) using specified primers, deoxyribonucleotide triphosphates (dNTPs), and a thermostable Taq DNA polymerase in buffer solution, through several cycles of denaturation, annealing, extension, final extension, and final hold reactions at varied temperatures (Griffiths 2014). By creating either specialised oligonucleotides that target certain fungal species or universal primers that amplify a variety of pathogens accompanied by sequencing, end-point PCR enables the precise diagnosis of fungal plant diseases. Nucleotide sequences can be compared to ex-type cultures recorded in the NCBI GenBank database utilising the Basic Local Alignment Search Tool (BLAST) analysis to identify each fungal isolate. The existence of a target shown by agarose gel electrophoresis guarantees the prevalence of known plant pathogenic fungi (Mirmajlessi et al. 2015).

The end-point PCR assay for *Phymatotrichopsis omnivora* detection, as well as a SYBR Green qPCR with a primer set PO2F/PO2R was highly sensitive (1 fg) in screening infected plants (Arif et al. 2013). The soil-borne fungus *Phymatotrichopsis omnivora* is responsible for root rots in important crops such cotton, alfalfa, soybeans, vegetable crops, and fruit and nut orchards. These assays may be used to predict the likelihood of disease in a field, assess the pathogen's survival during crop rotations with nonhosts, and examine fungal growth on resistant germplasm used in breeding programmes, among other things. The outlined assays may potentially be used in agricultural biosecurity regulations and microbial forensics (Arif et al. 2014).

Nested PCR

Nested PCR uses two sets of primer pairs for two rounds of PCR amplification to increase specificity and sensitivity. This technique also facilitates the use of general PCR primers in the initial round of PCR for amplification of several pathogens, accompanied by pathogen-specific primers in the second round (Bhat and Browne 2010). *Pilidiella granati* is responsible for pomegranate twig blight and crown rot, both of which are new to the pomegranate business. *P. granati* sensitivity and detection were improved by a nested PCR assay, which allowed for the determination of the causative agent even when only 10 pg of *P. granati* DNA was present in the sample (Yang et al. 2017). Great yam disease is caused by *Colletotrichum*

gloeosporioides, and eucalyptus dieback is caused by *Cylindrocladium scoparium* (Raj et al. 2013; Qiao et al. 2016), wherein this method was employed for detection. The sensitivity of detection with nested PCR could be raised by a factor of 10–1000 when compared to an end-point PCR experiment (Ippolito et al. 2002; Silvar et al. 2005). On the other side, because previously amplified samples are manipulated, nested PCR tests take a bit longer and have a higher chance of cross-contamination, which might also result in false-positive results (Raj et al. 2013). Actually, the use of nested PCR and end-point PCR as diagnostic tools is not advised due to the possibility of amplicon contamination.

Multiplex PCR

A multiplex PCR assay employs a single reaction mixture with multiple primer pairs to amplify multiple pathogens at the same time (Sint et al. 2012). Electrophoresis can then be used to separate and visualise the produced amplicons. Designing primers for the multiplex assay is essential for successful amplification, and particular sets of primers must have comparable annealing temperatures (Zhao et al. 2014). Using the multiplex PCR approach, a contemporaneous diagnostic assay has been developed to detect 12 fungi related with cranberry fruit rot. The ITS-LSU and TEF-1 gene sections were used to successfully identify the fungal infections *Allantophomopsis cytisporea*, *A. lycopodina*, *Phyllosticta elongata*, *Coleophoma empetri*, *Colletotrichum fioriniae*, *C. fructivorum*, *Fusicoccum putrefaciens*, *Monilinia oxycocci*, *Phomopsis vaccinii* (Conti et al. 2019), *Fusarium oxysporum*, *Bipolaris cactivora, Phytophthora nicotianae*, and *Phytophthora cactorum* are pathogenic fungi that threaten the cactus industry's export sector. This issue was resolved by using multiplex PCR assays. These quarantine fungal infections in grafted cactus were found to be detectable and identifiable using the diagnostic technique (Cho et al. 2016). Despite the fact that multiplex PCR assays are speedy and reliable, they can be costly and resource-intensive, and they have a lower sensitivity than other methods (Pallás et al. 2018).

4.3.2.2 Isothermal Amplification-Based Methods

A variety of methods, usually including the use of enzymes to take on the denaturing function at higher temperatures, enable DNA amplification to occur at a single, constant (isothermal) temperature. As opposed to PCR, which alternates between high temperatures for DNA denaturation and low temperatures for primer annealing and DNA synthesis, this does not require this. For instance, recombinase polymerase amplification (RPA) is comparatively new isothermal amplification technique (Piepenburg et al. 2006). RPA uses two primers, operates at 37–42 °C, and lasts for 10–30 min. Exponential amplification is produced by the process' cyclical repeating (Ereku et al. 2018).

Going beyond the laboratory has turn out to be a reality for molecular diagnostics, thanks to the development of isothermal amplification technologies, which allow nucleic acids to be amplified at a specific temperature without the use of thermo-cyclic equipment. Time and instruments no longer limit the amplification stage. Finding adequate ways for speedy and user-friendly plant preparations and detection

Table 4.2 List of the main isothermal amplification methods applied for fungal plant pathogen detection

Method	Target	Advantages	Disadvantages	References
Loop-mediated isothermal amplification (LAMP)	DNA/RNA	Rapid, isothermal, extremely sensitive, and relatively inexpensive	Designing primers can be challenging	Ammour et al. (2017), Aglietti et al. (2019), Wilisiani et al. (2019)
Recombinase polymerase amplification (RPA)	DNA/RNA	There is no need for an initial denaturation stage because the process is quick and isothermal	Long primers are required, sensitivity and specificity may differ	Ahmed et al. (2018), Gaige et al. (2018), Burkhardt et al. (2019)
Rolling circle amplification (RCA)	DNA/RNA	Isothermal, highly specific, and sensitive	Costly, and detection could be complicated	Rezk et al. (2019)
Strand displacement amplification (SDA)	DNA/RNA	Rapid and isothermal	Amplification of lengthy transcripts is inefficient	Song et al. (2018), Venzac et al. (2018)
Helicase-dependent amplification (HDA)	DNA	There is no need for an initial denaturation stage because the process is speedy and isothermal	High-level optimisation is required	Schwenkbier et al. (2015a, b), Wu et al. (2016)
Nucleic acid sequence-based amplification (NASBA)	RNA	Rapid and isothermal	The procedure is costly	Tsaloglou et al. (2011), Dobnik et al. (2014)

of amplicons following amplification are among the challenges to be solved. A summary of methodologies for in-field phytopathogen diagnostics based on several forms of isothermal amplification, as well as their benefits and drawbacks, are available (Table 4.2).

Recombinase Polymerase Amplification (RPA)

Isothermal RPA, first described in 2006 (Piepenburg et al. 2006), is a highly selective and sensitive isothermal amplification technology that operates at 37–42 °C, requires minimal sample preparation, and can amplify as few as 1–10 DNA target copies within 20 min. It has been used to amplify RNA, miRNA, ssDNA, and dsDNA from a wide range of organisms and materials. A growing number of papers describing the use of RPA are being published, and amplification has been done in solution phase, solid phase, and bridge amplification formats. RPA has also been effectively used with a variety of detection methods, including end-point lateral flow strips and real-time fluorescence detection (Lobato and O'Sullivan 2018). The recombinase-primer complexes are used in RPA reactions to scan double-stranded DNA and promote strand exchange at cognate locations, resulting in a better accuracy of recognition than PCR (Piepenburg et al. 2006). The

RPA produces a "single band" amplification product that is used for further molecular biology studies when contrasted to LAMP, another isothermal DNA amplification method (Iseki et al. 2007). As a result, the RPA assay might be used for routine field monitoring. In addition, RPA technology can be used in conjunction with a lateral flow dipstick to create a quick amplification and visual detection system.

A recombinase polymerase amplification (RPA) test was created to specifically detect *Bipolaris sorokiniana* based on the calmodulin gene sequences. Nineteen fungi related with wheat were used to test the RPA assay's specificity, and it was established that the detection limit for *B. sorokiniana* pure fungal DNA is 10 pg (Zhao et al. 2021). Several soil-borne fungal infections might be found immediately using the RPA test on artificially infected and field-collected plant tissues. These results imply that the RPA assay is a rapid and reliable technique for identifying soil-borne fungus.

Loop-Mediated Isothermal Amplification (LAMP)

Tsugunori et al. (2000) devised a nucleic acid amplification method. Because of its excellent specificity, simplicity, efficiency, and speed, this technique is widely employed. Isothermal amplification that relied on the precise design of four primers is referred to as LAMP (Notomi et al. 2000). To identify the six distinct sequences of the target DNA, LAMP employs two lengthy outside primers and two brief inner primers. The first inner primer, which has DNA sense and antisense sequences, will hybridise the target sequence, and DNA synthesis will start. The second inner and outer primers use the single-stranded DNA produced by the outer primer as a template to create a loop-structured DNA molecule. The outer primer also engages in strand displacement DNA synthesis. Two extra primers are annealed to these loops in modified LAMP. They speed up the reaction by up to 30 min by boosting it and producing additional DNA products (Nagamine et al. 2002).

This enables it a superior choice for plant pathogen diagnostics at the point of care in the field (Fukuta et al. 2013) and a different, trustworthy method for microbial pathogen detection and plant disease diagnosis (Ghosh et al. 2016, 2017). The LAMP assay's benefits and ease of use also include possibility of determining whether a reaction is positive or negative with the naked eye by spotting an elevation in turbidity or a change in colour, as well as the low cost of the equipment and chemicals needed for the reaction (Ghosh et al. 2017). At the same time, the lack of precision in primer designing and the large variety of primers to be chosen are the biggest roadblocks to this research gaining popularity. Nonspecific amplification and primer-dimer products result from using suboptimal primers and temperatures (Rolando et al. 2020). Complex multiplexing is another disadvantage of LAMP, which stems from the difficulty of designing two or more sets of primers. Nonetheless, a number of multiplexed LAMP (non-plant pathogen) systems have been created (Tanner et al. 2012).

Because this approach is less sensitive to inhibitors than PCR, it has been used to detect a variety of plant pathogens, including *Pythium aphanidermatum* from tomato roots (Fukuta et al. 2013), *Fusarium oxysporum* f. sp. *ciceris* (Ghosh et al. 2016) and *Rhizoctonia bataticola* (Ghosh et al. 2017) from disease-infested chickpea fields,

Didymella bryoniae from cucurbits (Tian et al. 2017a), and *Colletotrichum truncatum* from soybeans (Tian et al. 2017b). *Plasmodiophora brassicae* was detected in soil, roots, and seeds using a loop-mediated isothermal DNA amplification (LAMP) promising test with excellent sensitivity, precision, and simplicity. *P. brassicae* is a soil-borne protist pathogen that causes clubroot disease in cruciferous plants around the world (Yang et al. 2021). This method could detect *P. brassicae* in the soil with as little as 1 fg plasmid DNA or 10 resting spores. The LAMP was proved more sensitive than conventional PCR in detecting *P. brassicae* at lower levels in soil samples. Because resting spores of *P. brassicae* are the principal source of infection and can survive in soil for many years, the low level of detection allows forecasting models for clubroot prevalence (Yang et al. 2021).

Rolling Circle Amplification (RCA)

Using the isothermal amplification principle, rolling circle amplification amplifies circular DNA (RCA). By using a DNA polymerase with strand displacement activity (like 29 DNA polymerase), RCA implies spreading a single primer that has been annealed to a circular DNA template. The liberation of ssDNA is caused by the ability of newly synthesised DNA to displace already existing DNA through strand displacement activity. The long single-stranded DNA strand that comes from this enzymatic process of primer expansion and strand dislocation has a complementary sequence to the circular template.

For plant pathogen detection, rolling circle amplification has been frequently employed. Several approaches, such as RFLP and direct sequencing, have been employed in conjunction with RCA to efficiently identify and classify plant pathogens with substantially less work and cost than traditional technologies. By adding fluorescent dye to the reactions, naked eye visibility of the RCA product has been obtained for 40 *Fusarium* strains (Davari et al. 2012). For the detection of fungal infections, padlock probes have been ligated and then RCA has been established (Najafzadeh et al. 2011).

Nucleic Acid Sequence-Based Amplification (NASBA)

NASBA is an isothermal transcription-based amplification technique that is explicitly meant for single-stranded RNA or DNA sequence amplification. Compton (1991) was the first to introduce it, and it is conducted at 41 °C. The approach is highly suitable for RNAs such as mRNA, rRNA, tmRNA, or genomic RNA since reverse transcription activity is integrated into the amplification process (Deiman et al. 2002). NASBA, on the other hand, cannot amplify double-stranded DNAs that have not been denaturated (Yates et al. 2001). "Self-sustained sequence replication" (3SR) and transcription-mediated amplification (TMA) are other terms for it (Ghosh et al. 2019). The amplification power of NASBA is comparable to or better than that of real-time PCR tests, and it does not require a heat cycler (Loens et al. 2006). Additionally, because NASBA only requires brief reactions, has good sensitivity and tight control, and is unaffected by inhibitors, it is particularly appropriate for lab-on-a-chip systems (Honsvall and Robertson 2017). The usage in identifying fungal

infections in plants is extremely infrequent. It might, however, be used in the future to identify fungal diseases.

Helicase-Dependent Amplification (HDA)

HDA is probably the easiest techniques for isothermal nucleic acid amplification that closely resemble an in vivo DNA replication process by using a helicase to isothermally decompress DNA duplexes rather than heat to break away the nucleic acids, allowing labelled primers to anneal to the DNA template and lengthen under the activity of the polymerase, just like in conventional PCR. In 2004, Vincent et al. (2004) discovered this approach, which was later patented by Kong et al. (2007). Because of its simple reaction steps, helicase-dependent amplification has now become a common isothermal approach. Although it uses the same principle as PCR to amplify the target sequences with a pair of primers, the steps are simpler because there are no additional temperature cycling phases.

HDA paired with chip-based detection of *Phytophthora* species that are regulated has a lot of promise for on-site detection. Portable testing devices could be used in the field or at a place where a suspect plant needs to be evaluated with significant improvements. This can shorten the time between taking a sample of sick plants and getting a meaningful result by concentrating sampling, detection, and intervention. Isothermal nucleic acid amplification was developed to replace PCR, which requires a costly thermocycler, in order to achieve a potential field use. tHDA-based amplification and on-chip detection may be carried out in small and portable devices, allowing for on-site operation. Thermal cycling and time-consuming technical requirements are not required for the tHDA performance. Furthermore, the development of disposable, low-cost chips may hasten the availability of portable devices for chip-based DNA analytics in the near future.

4.3.2.3 Post Amplification Techniques

DNA Microarray

Schena et al. at Stanford University in California, USA, first introduced DNA microarrays in 1995 (Schena et al. 1995). A DNA microarray (DNA chip, gene chip, or biochip) is a collection of tiny DNA patches glued to a solid surface (typically glass) in predetermined positions (Bhatia and Dahiya 2015). It is a great tool for genetic study since it can display the expression of thousands of genes at the same time. It may apply thousands of nucleotides to a surface in an ordered array, allowing for simultaneous probing of thousands of distinct sequences (Hadidi et al. 2004; Barba and Hadidi 2008; Guigó 2013).

High performance and multiple diagnosis of diverse plant pathogens such as viruses, viroids, bacteria, and fungi have been made possible because of advancements in DNA microarray technology (Tiberini and Barba 2012; Musser et al. 2014; Nam et al. 2014; Krawczyk et al. 2017). Fungal pathogens are targeted with PCR primers and fluorescent probes like *Spongospora subterranea* (ITS region), *Rhizoctonia solani*, *Fusarium* sp. (TEF-1α), *Alternaria solani*, *A. alternata* (Alt_a1 gene), and *Colletotrichum coccodes* (TUB2) were used with

qPCR microarray technology in 48-well silicon microarrays (Nikitin et al. 2018). A unique microarray test (ArrayTube) that comprised marker genes ITS, TEF-1, and 16S rDNA with effective probes was used to find multiple sugar beet root rot pathogens such as *Aphanomyces cochlioides*, *Botrytis cinerea*, and *Penicillium expansum* (Liebe et al. 2016). On standard microscope slides, batch-based DNA microarrays can be produced quickly, easily, consistently, and affordably (Wöhrle et al. 2020).

DNA Macroarray

To make DNA macroarrays, on a nylon or nitrocellulose membrane, species-specific probes (15–30 bases of oligonucleotides) are arranged on well plates. Afterwards, probe hybridisation with PCR-generated and tagged target DNA sequences can be detected (Clark et al. 1999; Zhang et al. 2008). The manufacturing of membrane-based macroarrays requires simply a pin-tool. A 96-well microtitre plate-size membrane can hold over 1000 distinct detector oligonucleotides, and individually array can be cleaned and reused several times, albeit having a lesser throughput than a microarray (Zhang et al. 2008).

For the identification and detection of fungal and oomycete pathogens in agriculture, a range of macroarrays have already been created. The accuracy and sensitivity of these detecting systems have been demonstrated (Zhang et al. 2007). Over a hundred *Pythium* spp. can be detected using one of the most thorough DNA arrays (Tambong et al. 2006). Nevertheless, plenty of the macroarray investigations that have been published to date have only a few detector oligonucleotides for a specific set of pathogens. An apple disease detection array included 5 controls and 21 oligonucleotides specific for 7 fungal taxa and 1 bacterial target, whereas a tomato vascular wilt pathogen detection array had 3 controls and 10 oligonucleotides specific for 5 taxa (Sholberg et al. 2005). The array detection's slightly elevated capacity has yet to be realised. New vine decline (YVD), a complicated disease in grapevine induced by 51 fungal species and accountable for high mortality in young vineyards around the world, has been detected using DNA microarray (Table 4.3). This DNA array demonstrated to be a quick and specific approach for detecting and identifying the majority of YVD fungus in a single test, with the ability to be utilised in commercialised diagnostics (Úrbez-Torres et al. 2015).

4.3.2.4 DNA or RNA Probe-Based Assays

Because DNA-RNA probe assays are speedier and more sensitive than traditional diagnostics for plant diseases that require microbes culturing, molecular probe assays are rapidly replacing them. Molecular probe assays can be completed in a matter of hours or minutes, whereas culture procedures can take days/weeks. DNA and RNA probes are the most common types of molecular probes; however, cDNA probes and synthetic oligonucleotide probes can also be utilised for a variety of applications. There are four different types of probes that can be used in in situ hybridisation. Table 4.4 lists the probe types and their characteristics.

Table 4.3 Microarray vs macroarray—a overview

Feature	Microarray	Macroarray
Array platform	The glass slide	The nylon or nitrocellulose membrane
Size of the sample spots	Microarray sample spots are generally fewer than 200 μm in diameter, and these arrays can have thousands of dots	Sample spot sizes of 300 μm or larger are found in macroarrays
Advantage	The identity of the clone is revealed right away	Outcomes furnished in full-length clones
	Commercial arrays are available to buy	Obtaining clones in expression plasmids is simple
	Representation of rare genes can be more complete	Can create bespoke libraries to meet your specific requirements There is no need to know the order ahead of time
	Many companies are offering screening and data analysis services, as well as a simple screening process	Non-biased gene coverage on the array
	Array quality (particularly commercial arrays) is somewhat more stable	Filters are reusable
	To produce probes, you might start with total or mRNA	Screening techniques that are adaptable
	To compare two populations, a single hybridisation is used	
Disadvantage	For further research, full-length clones are required	Rigorously laborious
	Only one array can be utilised at a time	Each clone must be sequenced
	To accomplish hybridisation, you'll need a fluidic station and a reader	The quality of libraries and filters can differ
	Custom arrays are more costly than regular arrays	Rare transcripts may not be completely covered
	Gene coverage varies by company and EST database utilised for design	The amount of DNA at each place can differ from one filter to the next
	Sequence information is required to generate the array	Typically, each probe should only be screened once
	The quality of "home-spotted" arrays varies significantly	To compare two populations, sequential hybridisation was used
		Filters have a limited lifespan
		PhosphoImage displays are costly

In Situ Hybridisation (ISH)

In situ hybridisation is also termed as hybridisation histochemistry. It's a gold mine of information for recognising and counting fungi (Aslam et al. 2017). ISH is a technique for detecting and localising nucleic acid sequences in anatomically intact cells or morphologically conserved tissue slices. Single-stranded RNA probes, also known as riboprobes, are utilised in this approach. 35S is used to mark these probes.

Table 4.4 The information of probe types

Probe types	Advantages	Disadvantages
Double-stranded DNA (dsDNA) probes	Steady, accessible, easier to obtain	Self-hybridise, less sensitive, need denaturation before hybridisation
Single-stranded DNA (ssDNA) probes	Reliable, easier to maintain, more selective, RNase resistant, advanced tissue penetration, and no self-hybridisation	Time-consuming and expensive
RNA probes (riboprobes)	RNase has improved temperature constancy, tissue penetration, and specificity while reducing background noise	Sensitive to RNases
Synthetic oligonucleotides	Inexpensive, robust, readily available, easily dealt, more specific, RNase resistant, greater tissue penetration, and repeatability	Acquire nucleotide sequence information

Northern blots and in situ hybridisation are very similar. Both of these rely on the hybridisation of tagged DNA/RNA probes to homologous mRNA sequences. The use of beginning material differs between these two procedures. Tissue digest is utilised as the starting material in northern blots, while histological sections are used in in situ hybridisation. Regardless of whether direct hybridisation is used or not, signal hybridisation identifications are most effective following fungal growth or biological amplification (Jensen 2014).

The radioactive isotopes 35S, 125I, and 32P are commonly used to label probes because they are extremely sensitive and easy to quantify for detection. Non-isotopic probes can be labelled using biotin, digoxigenin, tyramide, alkaline phosphatase, or bromodeoxyuridine. Signal detection techniques include photography, autoradiography along with X-ray film, liquid emulsion, and microscopic techniques (Corthell 2014). *Puccinia horiana* isolate PA-11, *Uromyces transversalis* isolate CA-07, and *Phakopsora pachyrhizi* isolate Taiwan 72-1, which infect *Chrysanthemum morifolium*, *Gladiolus hortulanus*, and *Glycine max*, were identified as rust pathogens using the ISH approach (Ellison et al. 2016). Several *Fusarium oxysporum* formae speciales were genetically engineered with two marker genes and stained with fluorochrome-labelled probes in in situ hybridising transcripts of the marker genes (Nonomura et al. 1996).

Fluorescence In Situ Hybridisation (FISH)

FISH is a type of ISH that uses fluorescent probes to connect with particular chromosomal regions in order to show sequence complementarity. FISH and all other in situ hybridisation techniques share the same fundamental principles; the only difference is that one uses a fluorescent probe to detect specific nucleotide sequences across cells and tissues (Hijri 2009). In plant disease diagnosis, fluorescent in situ hybridisation (FISH) is a relatively new and creative method. It integrates the selectivity of DNA sequences with the accuracy of fluorochrome-based detection techniques (Hijri 2009; Cui et al. 2016). To identify DNA or RNA sequences in cells

Table 4.5 Advantages and disadvantages of ISH and FISH

Feature	ISH	FISH
Advantage	On the same tissue, variety of new hybridisations can be performed. Tissue libraries can be made and preserved in the freezer for later use. The most specific and efficient method of probing is with riboprobes (Aslam et al. 2017)	FISH/s main strengths include reproducibility, sensitivity, specificity, accuracy, and rapidity (Bozorg-Ghalati et al. 2019). It also has the ability to provide data on resolution, morphology, and pathogen identification in combined species specimens (Frickmann et al. 2017)
Disadvantage	The expense and hazards of radioactive probes, as well as the complexity of identifying targets with low DNA and RNA quantities, are the major drawback of ISH (Jin and Lloyd 1997)	False-positive autofluorescence outcomes are a major stumbling block that lowers test specificity (Moter and Göbel 2000)

or tissues, FISH techniques employ DNA or RNA probes that are fluorochrome-labelled explicitly or implicitly (Shakoori 2017). Using wide field epifluorescence or confocal laser scanning *Sclerotium rolfsii* imaging, fluorescently mono-labelled oligonucleotide probes are hybridised to the ribosomal RNA (rRNA) of microbial cells in classical FISH (Lukumbuzya et al. 2019). Plants infected with a pathogen will have rRNA sequences peculiar to that pathogen. FISH allows for the accurate determination of the information that RNA provides (Fang and Ramasamy 2015). The soil-borne pathogen *Sclerotium rolfsii* causes southern blight, which damages tomatoes. FISH approach, which used an oligonucleotide probe stained with cyanine dyes Cy3 and Cy5, was efficient in detecting soil smears in a DNA isolation with 0.06 pg (Milner et al. 2019) (Table 4.5).

4.4 Use of Next-Generation Sequencing (NGS) in Plant Pathogen Detection

Due to its ability to target many unique signature loci of pathogens in a diseased plant metagenome, next-generation sequencing (NGS) has potential as a diagnostic tool. NGS holds a lot of promise for detecting key eukaryotic plant diseases (Espindola et al. 2015). NGS was first used for genome sequencing, supplementing and later substituting the classical genome sequencing method, which included cloning of DNA fragments, Sanger sequencing and genome walking to sequence individual clones, and compilation of the sequenced clones. New NGS platforms and versions have been created on an exponential scale as sequencing chemicals, computer hardware and software, as well as computational capability have advanced. Different NGS systems have their own set of benefits and drawbacks (Tsang et al. 2021).

The baseline genotypes, which might be used to learn the biology and evolution of some other species' genomes, have been sequenced. An instance of a

circumstance in which the target cannot be properly defined is the appearance of a novel pathogen. The full genome of the pathogenic organism can be sequenced using NGS without the requirement for specialised primer pairs or PCR amplification because it does not require prior knowledge of pathogen sequences (Hadidi et al. 2016; Malapi-Wight et al. 2016). Third-generation sequencing is a development in single-molecule sequencing technology, which also has advantages over second-generation sequencing techniques among NGS technologies (Schadt et al. 2010). The time needed to collect and analyse the massive volumes of sequence data is the largest drawback of NGS (Espindola et al. 2015). Inadequate RNA production and/or integrity, RNA stability, and contamination with DNA, salts, or chemicals are typically barriers to the development of next-generation technologies (Cortés-Maldonado et al. 2020). Despite how quickly and easily the sample can be gathered, NGS analysis requires bioinformatics and mycological skills; as a result, accurate bioinformatics analysis knowledge is essential to prevent misinterpretation.

4.5 Conclusion and Future Challenges

We glanced traditional methodologies as well as currently available advanced technologies for detecting and identifying fungal pathogens causing soil-borne diseases. The purpose of this chapter was to highlight the developments in the field of advanced detection technologies. Plant pathogen diagnostic techniques have made a substantial contribution to our capacity to detect and examine pathogens in the lab and, more subsequently, in the field. Existing molecular procedures provide consistent sensitivity and are generally faster than traditional techniques. Monitoring and the implementation of novel disease control measures enable a thorough understanding of pathogenicity variables, as well as fast and effective detection of fungal infections down to the species level. Furthermore, early detection of resistance levels in soil-borne fungus in a field would aid farmers in developing effective resistance management plans to combat disease.Nevertheless, because no single approach meets all, if not the majority, of the developing criterion for faster, more effective, repeatable, and sensitive outcomes, there is still a significant knowledge gap in this sector.

Quantitative PCR has been frequently utilised to quantify and separate causal agents when the sample load is too small to detect using other PCR-based methods. Amplification techniques are showing promise in the field of fungal disease detection, allowing for the identification of pathogens such as *Alternaria* spp., *Colletotrichum* spp., *Fusarium* spp., *Verticillium* spp., *Botrytis* spp., and others that cause a variety of devastating soil-borne plant diseases. The ability of NGS to sequence fungal genomes without prior knowledge of the pathogen's sequence makes it useful for discovering new and emerging illnesses. The molecular methods described in this chapter for diagnosing fungal plant diseases are precise, effective, lab-based, and require high-end equipment. On the other hand, mycology and bioinformatics knowledge are intended to prevent inaccurate portrayal of the outcomes of molecular biological study. By integrating molecular methodologies

with other novel technological advancements, point-of-care testing for fungal illness diagnosis should become commonplace. Scientists have been tasked with developing practical molecular diagnostics for crop diseases. We anticipate that this will start to alter in the upcoming years.

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