Technological Advances in Phytopathogen Detection and Metagenome Profiling Techniques

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Abstract

The use of advanced molecular methods in plant pathology and applied microbiology has necessitated for more accurate, rapid detection and identification of plant pathogens. This is particularly significant given accelerated emergence of virulence that leads to increased prevalence of plant pathogens. Thus, the capacity to contain plant pathogens and ultimately disease progression is key to ensuring crop biosecurity and overall food security. Of recent, research on pathogens utilizes a holistic approach focusing on elucidating growth dynamics within the entire biome rather than studying individual or closely related isolates in unison. This has advanced knowledge and information of microbial ecosystem within natural environments in the twenty first century. Applied technological platforms used for rapid detection and profiling microbial biomes in this regard include digital PCR, pyrosequencing, Illumina, DNA microarray and barcoding, Ion torrent, and nanopore. These technologies have been applied in various fields including human health and medicine, marine and animal biology, crop production and water quality research, to mention but a few. Although much has been done and achieved through the development of several technologies, more accuracy is required to circumvent the shortfalls still experienced. This includes integrating existing methods with new applications such as viability PCRs and microbial viability testing. Hence, this review provides critical analysis of some widely used latest technologies in rapid detection and identification of plant pathogens, and profiling plant associated microbiomes that reveal growth dynamics and population diversity. The advantages and limitations of the technologies are also discussed.

Introduction

The phyllosphere is fundamental to plant health and contributes towards a better understanding of microbial dynamics. Like any other plant, the phyllospheric microbial consortia harbor neutral or beneficial [1] and phytopathogenic [2] microorganisms. There are several beneficial inhabitants of the phyllosphere possessing antagonistic properties against pathogens. On the other hand, pathogens within the biome await opportunity to invade the plant tissue or system pre or postharvest. In some instances, infection occurs during flowering [3], thereafter, the pathogen remains dormant until proliferation is triggered by favorable conditions during ripening [4]. It is important to note that these plant pathogens and subsequent disease outbreaks are regularly emerging and spreading to new environments. For instance, there have been first reports of cabbage leaf curl virus and *Meloidogyne haplanaria*

in Ecuador [5] and Florida (USA) [6], respectively. These diseases could severely impair produce quality leading to a major cause of economic loss for the crop production industry.

Overall losses attributed to plant diseases in developing countries have been reported to be over 40% of the total production [7]. For instance, gray mold caused by *Botrytis cinerea* on table grapes is responsible for 20% postharvest loses globally, amounting up to 100 billion Euros per year [8]. In Africa, two viral diseases, cassava mosaic disease and cassava brown streak disease, are amongst the causes of yield reduction of cassava. These diseases are estimated to cause annual losses of about US$1 billion [9]. In apples *Penicillium expansum* can cause decay losses estimated between US$4.5 and US$5 million annually [10].

To reduce such losses, several disease control strategies such as fungicides [11], essential oils [12] and other biocontrol formulations have been developed. It is important to note that the current trend is focused on developing alternative applications to replace fungicides. However, it remains imperative to develop rapid diagnostic platforms to accurately detect disease causal agents as well as elucidating microbial profiles to reveal pathogen biome relationships. This will, among others, help develop disease prediction models and alternative control applications.

In this context, recent advances in bioinformatics and metagenomics have provided more rapid and highly sensitive protocols than conventional microbiological methods [13]. Most of these culture independent and high throughput techniques tend to focus on the whole biome rather than studying individual or closely related taxa [14]. These recent molecular technologies have been used to detect phytopathogens [15] and profile phytobiomes by fingerprinting or sequencing [16]. These nucleic acid based techniques include PCRs (such as digital droplet), denaturing gel gradient electrophoresis, terminal restriction fragment length polymorphism (T-RFLP), DNA microarrays, ion torrent, 454 pyrosequencing, Illumina MiSeq and nanopore. Using these technologies enable the study of the natural microbiome to reveal more in depth dynamics of the plant microbial interactions. Therefore, this paper will discuss methods, including latest technologies, used to detect plant pathogens and profile plant microbiomes. The application and limitations of the methods will also be discussed.

Digital Droplet PCR

Several studies have used conventional [17], multiplex [18] and real time PCR [19] to detect and verify identities of plant pathogens. Of recent, a highly sensitive droplet digital PCR was developed which uses limiting dilutions and Poisson statistics to allow precise quantification of target nucleic acids in a sample [20]. This concept, first described by Sykes et al. [21], determines total concentrations of nucleic acid copies by partitioning a sample into thousands to millions of small reactions within water in oil droplets before PCR amplification [22]. This technology does not require the use of calibration standards, and quantification is more resolute to fluctuating Cq values compared to qPCR [22].

The ddPCR was initially used in clinical research [23, 24], and until now, not common in plant pathology. However, Dreo et al. [25] conducted a case study wherein the ddPCR technology was optimized to detect and quantify *Erwinia amylovora* and *Ralstonia solanacearum* from bacterial suspensions and in plant material. The ddPCR detected low target concentrations of *R. solanacearum* in all plant material samples tested compared to only 45% effectiveness recorded with the qPCR. However, ddPCR and qPCR revealed similar levels of sensitivity and quantification range of *E. amylovora* between ddPCR and

qPCR. The rapid detection and total quantification of *E. amylovora* and *R. solanacearum* is of economic importance because these are quarantine pathogens infecting several species of *Rosaceae* and *Solanaceae* families, respectively.

In a recent study, ddPCR detected and quantified DNA copies of *B. cinerea* on asymptomatic table grapes at different phenological stages (flowers and, pea size and mature berries) [15]. The pathogen, *B. cinerea*, was not detected with other screening methods such as conventional culture techniques. Surprisingly, 454 pyrosequencing analyses of the same samples did not reveal the presence of *B. cinerea* [26], showing that ddPCR was more sensitive compared to the former. The importance of more sensitive methods for early detection of *B. cinerea* is necessary in developing disease prediction models and increasing efficacy of control measures.

Palumbo et al. [27] developed a simultaneous species specific ddPCR method for quantification of *Aspergillus* species in soils collected from raisin vineyards. Their results showed a dynamic population of *Aspergillus* species within and between vineyards. For instance, at one sampling site between 2013 and 2014, *Aspergillus niger* comprised 8.3–88% and 24–73%, *Aspergillus welwitschiae* comprised 0.5–20% and 2.2–50%, and *Aspergillus carbonarius* comprised 1.1–78% and 0–28%, respectively. Thus, dispersal of mycotoxigenic fungi from soil onto plant carposphere is possible. The ddPCR has therefore, provided a platform to control preharvest mycotoxin contamination of raisin and other crops.

Denaturing Gel Gradient Gel Electrophoresis

Characterizing the diversity of microbial populations using denaturing gradient gel electrophoresis has been achieved in several study areas [28,29,30]. Profiles of bacterial populations inhabiting casing material used in the production of mushrooms (*Agaricus bisporus*) were revealed using DGGE [30]. The casing samples used in the study were collected at three phases of mushroom growth i.e., casing, pinning and harvesting. The results of the study indicated higher bacterial diversity during pinning and harvesting than at casing. Several of the species identified belong to the genus *Pseudomonas* including *P. putida*, *P. fulva* and *P. fluorescens*. On the other hand, significant quantities of bacterial populations were not identified, signifying the need for more studies in the area. In addition, a holistic approach to revealing both bacterial and fungal profiles is required to fully unlock microbial dynamics in mushroom production. This will help understand growth of species antagonistic to the mushroom, improve management practices and improve decision making regarding disease control.

In another study conducted in Spain, DGGE was used to investigate fungal diversity in grape must and wine fermentation [29]. The study reported the detection of fungal species such as *Botryosphaeria dothidea*. However, DGGE was not able to detect species such as *Hanseniaspora opuntiae* but sequences belonging to the fungi were revealed using 454 pyrosequencing of the same samples. Thus, the study proved that the sensitivity of next generation profiling techniques supersedes that of DGGE. However, quantities of active microbial populations require the use of RNA because genomic DNA can only imply presence rather than pathogen activity.

DNA Microarray

Microarray, which uses several approaches, is a technology used for massive parallel sequencing. One of the many approaches applied by this technology involves immobilization of the sequence target in a microarray format, then using a very large set of short, labeled probes, the target is hybridized [31]. Thereafter, the hybridization pattern is examined and original pattern of DNA sequence is computed. This technology has been used for gene expression in several studies [32, 33]. Pham et al. [34] applied micro array to characterize the genes expressed by *Phytophthora capsici* during the infection cycle. Such applications have been confirmed effective in identifying critical infection phases that can be manipulated to inhibit pathogenesis and reduce infection rate.

Despite all the advances that next generation sequencing (NGS) has made, researchers still use microarrays [34]. Over the past two decades the technology has been used intensively by researchers for analysis of virulence factors [35], microbial community [36] and whole genome analysis [37]. This application is generally considered less complex, easier to use, and less laborious with regard to sample preparation. However, the limitation of this method is that the organism must be cultured and it is more accurate and effective under low density systems [38].

Ion Torrent

Ion torrent uses a semiconductor sequencing that is based on the detection of hydrogen ions released during DNA polymerization. Leboldus et al. [39] used a two enzyme restriction associated DNA genotype by sequencing method adapted for Ion Torrent sequencing technology to identify and characterize genotypes of fungal species from *Pyrenophora teres f. maculate* and *Sphaerulina musiva*. Genotype by sequencing can generate millions of reads; however, the method does not reduce the complication of the fungal genome. Leboldus et al. [39] reiterates that despite generating large sequence reads, the most minimal amount of coverage was achieved for many regions of the genome.

The semiconductor sequencing implemented in the Ion Torrent Personal Genome Machine was utilized for fungal community analysis to reveal high taxonomic diversity of ITS1 nuclear encoded ribosomal RNA of the endophytes in *Eucalyptus grandis* [40]. This study reported sequence reads over 2.3 million, despite using high quality filtering that discarded majority of the observed reads. It demonstrated the ability of semiconductor sequencing in recovering plant associated fungal biomes. However, the authors concede that limitations of the technology underestimate fungal diversity due to the presence of unknown taxonomic affiliations. Hence, other NGS technologies supersedes Ion torrent in this regard.

454 Pyrosequencing

Pyrosequencing technique analyses the hypervariable region of the bacterial 16S rRNA [14, 41, 42], and either the ITS1/ITS4 [43] or D1/D2 [44] variable regions are targeted for fungal populations. This high throughput metagenomic method is based on sequencing by synthesis principle [45]. Utilization of the 454 pyrosequencing to generate gene sequences has been used to reveal microbial populations in several samples that include soil [46], water and on vegetable carpoplanes [14, 42]. Of recent, studies elucidating microbial communities on fruit surfaces using pyrosequencing have been conducted [26, 47].

Abdelfattah et al. [47] conducted a study on phyllospheric fungal diversity on strawberry at different phenological stages and the effect of management practices thereof. The results of the study revealed 218,164 high quality sequences which were assigned to 316 operational taxonomic units (OTUs). The most prevalent species were *Botrytis* and *Cladosporium* more than all of the other identified genera combined, representing 70–99% of the relative abundance. Thus it was not surprising to note that the study also reported high incidence of gray mold infections in the field during the sampling period. Other sequences belonging to known plant pathogens *Fusarium equiseti* and *F. avenaceum* were detected, while their roles on strawberry carpoplane are unknown.

Carmichael et al. [26] also characterized fungal communities at different developmental stages in table grapes revealing higher diversity (2,035,933 OTUs) compared to that reported by Abdelfattah et al. [47]. Both studies reported the dominance of genetic sequences belonging to *Cladosporium*, but in contrast, Carmichael et al. [26] detected *Alternaria* as the most dominant genera. Both studies were conducted at commercial vineyards under normal management practices, noting reduced fungicide efficacies. According to Carmichael et al. [26] reduced fungicide efficacy could be attributed to a conducive microclimate prevailing at the center of the vine which favor pathogen proliferation.

In New Zealand, ecological and regional differences of fungal communities associated with grapes were revealed using pyrosequencing of the D1/D2 region of the 26S locus [43]. The authors opted to amplify the D1/D2 region over the commonly amplified ITS locus to discriminate between fungi, arguing that the former does not display the length polymorphism, thus, generating more reliable alignments. According to Taylor et al. [44], rarefaction analyses indicate that Hawke's Bay had the highest species richness, followed by West Auckland, Marlborough, and Central Otago, respectively. All regions differed in terms of diversity, each with signature species which may influence the regional uniqueness of wines produced.

This technology can help predict the risk of resistance breakdown in plants by estimating frequencies of virulent isolates [43]. In addition, pyrosequencing provides a holistic insight into the possible survival and persistence of pathogens that have potential to alter pre and postharvest quality and safety fresh produce [48]. As a result, understanding these microbial dynamics could improve the development of intervention strategies and provide a more durable approach to reduce spoilage. Despite major achievements of using pyrosequencing, two inherent challenges exist. These are related to de novo sequencing of polymorphic regions in heterozygous DNA and difficulties in enumerating incorporated nucleotides in homopolymeric regions [49]. It is important to note that pyrosequencing does not indicate microbial activity but presence alone. Therefore, the detection of genomic material does not indicate pathogen viability; nonetheless, presence of pathogenic DNA implies activity at one point or another.

Illumina

Illumina is the current leader of modern sequencing technology which offers the highest throughput compared to all NGS platforms at the lowest per base cost [50]. The technology has allowed for sequencing across a broad array of applications in genomics, transcriptomics, and epigenomics. Illumina MiSeq platform was used to correlate microbial alterations with occurrences of a postharvest disease, stem end rot on mango [51]. Mature mango fruit was harvested**,** stored for three weeks in the cold (5 or 12 °C), and thereafter at 20 °C. In this

study, the V4 region of the 16S rRNA genes and the ITS 1 loci were amplified to reveal bacterial and fungal biomes, respectively. The results indicate microbial alterations at harvest, during cold and shelf life storage. For instance, genetic sequences belonging to fungal taxa such as *Sporidiobolaceae* prevailed at harvest, reducing transiently during cold storage and not detected after shelf life storage [51]. Interestingly, the opposite was noted for bacteria such as *Chitinophagaceae* that was not detected at harvest, but was the second most abundant at shelf life storage. The emergence of this chitin producing bacteria was possible due to increased levels of, first, some pathogenic fungi (i.e., *Alternaria alternata* and *Lasiodiplodia theobromae*) and, later stem end rot pathogens. In this study, Illumina MiSeq technology revealed that several conditions affect microbiomes on mango, thus influencing occurrences of stem end rot. However, this platform can be used to further explain relationships and compositional variations between fungal [52] and bacterial populations in mango and indeed other fruits. Ultimately, such knowledge can inform development of more effective applications against targeted pathogens and, in turn, enhance disease control and management.

In a previous study, spatial and compositional variations in the fungal communities of organic and conventionally grown apple (at retail) were studied using Illumina MiSeq [53]. Dominant phyla colonizing apples included Ascomycota (69.3%), Basidiomycota (29.5%) and unidentified fungi (0.8%). Compositional disparities between organic and conventionally grown include higher relative abundance of *Ascomycota* in samples of the former, while *Basidiomycota* were more abundant in samples from the latter. To determine spatial variations, biomes from different parts [stem end (SE), calyx end (CE), wounded flesh (WF), and peel (PE)] of the same apples were analyzed. The results indicated significant differences of fungal populations on both organic and conventionally grown apples [53]. Some of the factors influencing these disparities include uneven distribution of nutrients and water; exposure to UV light; and topography of the fruit surface. For instance, the sunken areas of the apple, stem and carlyx end, shields microbes from UV light. Despite the recent advances in genomic based analysis used for identification and classification of microorganisms, the presence of unidentified fungi signifies that the biomes are yet to be fully characterized.

This could be due to limited genetic variations within the analyzed gene regions, hence, more work is required to identify new species and update current databases. More specifically, sample loading challenges resulting in overlapping clusters and poor sequence quality, and sequence complexity requisites have been reported to impede absolute success of NGS platforms, including Illumina [54]. In addition to the inability of NGS to discriminate alleles to parental homolog, the reliance of the technology in PCR leads to complexities with regions of extreme GC%. Notwithstanding the achievements of NGS, more has to be done to deal with the shortfalls mentioned herein.

Future Prospects in Plant Microbial Profiling

Shortly after the latest NGS technology was introduced, nanopore sequencing was developed. This technology is devoid of PCR amplification, sequence in real time, produce long reads and capable of single molecule sequencing [55]. Bronzato Badial et al. [56] attests that several existing technologies used for rapid molecular detection of plant pathogens require previous knowledge of the causal agent involved, as the assays are fundamentally targeting specific pathogens. The nanopore technology and other NGS platforms are capable of identifying pathogens that are either not known to be present in the sample and/or those not previously sequenced.

Of recent, Chalupowicz et al. [57] utilized nanopore technology for the diagnosis of inoculated plant pathogens from symptomatic tissues. The majority of the plant pathogens (e.g., *Penicillium digitatum* and *Pseudomonas corrugate*) were identified, while there were few unidentified disease agents (e.g., tomato and cucumber leaf spots). The authors admit that not all the pathogens, especially fungi, have the complete genome available in the reference database, and in addition, the majority of the fungi genomes present in the reference database are not plant pathogens. This indicates a need to improve fungi genome sequences in reference databases used for nanopore platforms to enhance sequence based diagnosis of plant pathogens. Chalupowicz et al. [57] also explored multiple sequencing of plant tissues in a single run and reported increased number of unidentified reads owing to diluted pathogen DNA. More optimization has to be done to avoid sequencing of non target sequences such as host endophytic/epiphytic microorganisms present in the diseased tissue. Ultimately, the study did not recommend sequencing of several samples for plant pathogen diagnosis. The study concludes that nanopore technology is rapid, shortens process diagnosis, provides results in real time and does not require in depth bioinformatics expertise.

In another study that used a nanopore technology, the Oxford MinION, was able to sequence metatranscriptome of plant and insect tissues infected with either *Candidatus Liberibacter asiaticus* or plum pox virus [56]. This marks the development of a simultaneous rapid detection and accurate diagnostic tool for plant pathogens and their vectors. Although the assay was able to detect and discriminate the pathogen and the vector in a complicated matrix, plant derived datasets had more total number of reads compared to insect datasets. The authors reiterate the known difficulties of acquiring RNA from insect samples and an irregular distribution of the pathogen in the insect host. The latter is confirmed by Fagen et al. [58] who focused on the abundance of *Ca. L. asiaticus* in an insect. This technology is thus a promising rapid and accurate tool to identify multiple unexpected pathogens pending improvements into a cost effective assay. In addition, this technology potentially supersedes other NGS platforms as handling of large datasets may be avoided.

One of the important future aspects in plant disease diagnostics and phytobiome profiling is the inclusion of viability PCR and molecular viability testing to address traditional PCR's inability to discriminate DNA associated viable cells from inactivated or free genomic fragments. In a recent study, viability PCR was used to selectively amplify viable bacterial cells using a cell membrane impermeable dye, propidium monoazide [59]. The dye modifies only exposed DNA from dead cells which are excluded during PCR amplification, followed by Illumina sequencing targeting the V3–V4 region of bacterial 16S rDNA. The synergy between viability assays and NGS can have a significant application in applied microbiology and pathogens causing diseases to plants.

Conclusion

The advent of advanced molecular based techniques, particularly NGS, has enabled plant microbiome researchers to study biological systems at a holistic and in depth level. The evolution of these technologies has transitioned and diversified research leading to increasing number of sample preparation methods. Currently, Illumina is the leading NGS platform, offering the highest throughput and the lowest per base cost. As costs are lowered, large scale sequencing of plant microbiomes will increase and advance our understanding of plant pathogen dynamics, improve disease diagnosis, and ultimately improve plant health pre and postharvest. It is proving difficult to develop methods devoid of setbacks, hence, one of the latest nanopore platforms cited for high error rate and lacking the ability to sequence the

same strand multiple times [50]. Several propositions such as (a) multiple recognition points for DNA sequence determination, and (b) increasing overall read length and throughput. In future, plant microbiome studies have the potential to further use the latest sequencing platforms such as Illumina and nanopore, to mention but a few, as well as combine these protocols with new dimensions to selectively target and discriminate viable and non viable DNA, without depending on traditional culture methods which has many limitations.

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