Annex 1

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Review Article

Global Challenges in Identifying Plant Pathogenic Fungi: An Overview with Suggestions for the Way Forward

Monika C. Dayarathne [a], Kasun M. Thambugala*[b], Dinushani A. Daranagama [c], Amin U. Mridha [d], Asha J. Dissanayake [e], Lakmali S. Dissanayake [f], Sajeewa S.N. Maharachchikumbura [e], RMCS Rathnayake [c], Saisamorn Lumyong [g] and Sinang Hongsanan*[g,h,i]

[a] Postgraduate Institute of Agriculture, Old Galaha Road, Peradeniya, Sri Lanka
[b] Genetics and Molecular Biology Unit, Faculty of Applied Sciences, University of Sri Jayawardenepura, Gangodawila, Nugegoda, Sri Lanka
[c] Department of Plant and Molecular Biology, Faculty of Science, University of Kelaniya, Kelaniya, Sri Lanka
[d] Daffodil International University, Ashulia, Savar, Dhaka, Bangladesh
[e] School of Life Science and Technology, University of Electronic Science and Technology of China, Chengdu 611731, People's Republic of China
[f] Engineering Research Centre of the Utilization for Characteristic Bio-Pharmaceutical Resources in Southwest, Ministry of Education, Guizhou University, Guiyang, Guizhou Province 550025, People's Republic of China
[g] Research Center of Microbial Diversity and Sustainable Utilization, Faculty of Sciences, Chiang Mai University, Chiang Mai 50200, Thailand
[h] Department of Entomology & Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand
[i] Guangdong Provincial Key Laboratory for Plant Epigenetics, Shenzhen Key Laboratory of Microbial Genetic Engineering, College of Life Science and Oceanography, Shenzhen University, Shenzhen 518060, People's Republic of China

*Author for correspondence; e-mail: kasun@sci.sjp.ac.lk, sinang333@gmail.com

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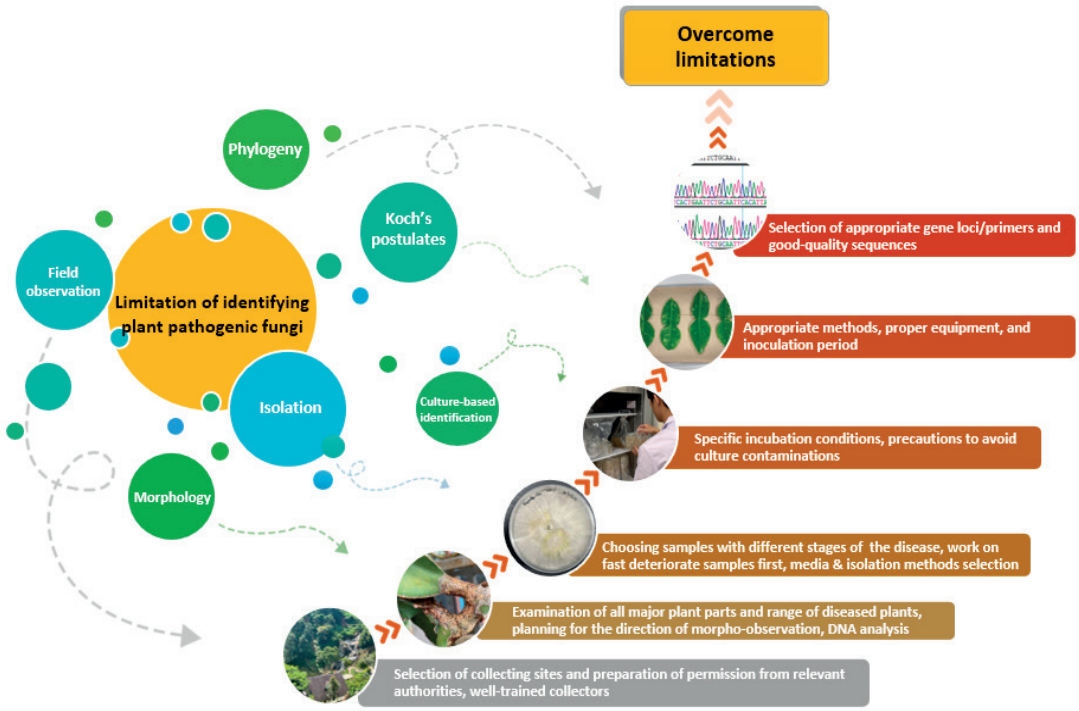
ABSTRACT

Fungal plant diseases are responsible for major crop and postharvest losses worldwide, with a significant socio-economic impact on many individuals who depend on an agriculture-based economy. This review discusses the major constraints in identifying plant pathogenic fungi (severely destructive on important crops) in sample collection, field, and morphological observations, fungal isolation, obtaining pure cultures, applying Koch's postulates, DNA barcoding, phylogenetic analyses, and naming fungi (under "One fungus, One name" concept) based on selected case studies. Additionally, available strategies/methods to overcome those limitations up to date are also discussed. Ultimately, we proposed suggestions to minimize those limitations.

Keywords: DNA barcoding, fungal taxonomy, morphology, pathogens, plant fungal diseases



Graphical abstract: Overview of limitations and overcome limitations in identifying plant pathogenic fungi.

1. INTRODUCTION

Most plant diseases caused by pathogenic fungi are responsible for major economic losses in the agricultural industry worldwide [1,2]. Various fungal pathogens can infect plants and the appropriate amount of inoculum for infection is accompanied by variations in environmental conditions such as temperature, humidity, soil, water, air, and host susceptibility. The susceptible plant species or crop varieties may exhibit visible morphological symptoms in or on the tissues where the infection is initiated. If the fungal pathogen can find favorable conditions for further development, systemic symptoms are induced in tissues or organs far away from the infection sites. When the symptom of the infection is not expressed externally, it is termed latent infection. Some fungal pathogens infecting unripe fruits do not express any visible symptoms, as they remain dormant. Detection of fungal pathogens refers to the process of establishing the consistent presence of a particular target organism within the plant or in environments, irrespective of the development of visible symptoms in the plant suspected to be infected by the fungal pathogens in question [3,4].

The main goal of plant disease diagnosis is to identify pathogens via a fast, accurate, and reliable method. Fundamental methods aiding in the detection of relevant fungal pathogens mostly rely on characteristics with cultural approaches that require extensive time, labor, and enough fungal taxonomy knowledge [5]. Fungi as microorganisms with distinct metabolisms require specific growth and reproductive requirements. Hence, an appropriate growth media is needed to grow the targeted pathogen. These approaches give

rise to unreliable results due to misidentification. As well, experts and specialists with a practice in fungal identification were required for effective results via these conventional approaches [6,7]. Detecting fungal pathogens early stage is crucial to reduce or prevent disease spread and establish effective control measures. DNA-based and serological methods provide essential tools for accurate plant disease diagnosis, in addition to the traditional visual scouting for disease symptoms [1]. However, there are various constraints in each fungal plant pathogen identification such as sample collection, field observations and recording details, morphological studies, isolation, culturing, serological and phylogeny-based identification, and applying Koch's postulates. Due to the limitations of conventional methods, molecular techniques came into use for investigation of identification and classification problems [8]. A considerable variation of molecular methods is increasingly becoming reliable tools in fungal pathogen identification. These techniques comprise nucleic acid-based probe technology, polymerase chain reaction (PCR) technology and immunological methods [7]. Earlier methods were based on phenotypic characters, while later relied on genotypic characters, which gave fast, precise, effective, and potentially more accurate results [9,10,7]. However, each technique has limitations, and this review further discusses them.

Objectives of this study are to (i) review the major constraints in identifying destructive plant pathogenic fungi for major important food crops under the following aspects: field observations, details recording, sample collection, morphological observations, fungal isolation, obtaining pure cultures, application of Koch's postulates, serological methods, DNA barcoding, phylogenetic analyses, and naming fungi (under one fungus one name concept) based on selected case studies (ii) discuss available strategies/methods to overcome those constraints (iii) provide important suggestions and precautions to minimize the above limitations.

2. LIMITATIONS IN FIELD OBSERVATIONS, SAMPLE COLLECTION AND RECORDING

The collection of appropriate samples in sufficient quantity is one of the most important practices in the process of plant-pathogen identification. Prior field observations and recording details must be performed together with the sample collection to analyze the pathogen and disease severity. However, several limitations in field observations, sample collection and recording details need to be addressed.

2.1 Field Observation Restrictions

Some of the occurrences of symptoms may not be open to observation and sample collection. Special permissions to access state crop fields are required in many counties as only authorized personnel can access those field investigations. For instance, permits to collect plant material in the USA should be obtained at a USDA Forest Service District Office. Furthermore, the permit types vary depending on collection needs and Forest Service personnel will identify whether a permit is required and, if so, what type. In addition, Forest Service units may also provide specifications such as specific locations where the collection is not permitted, a permit area map, a list of rare plant species, look-alikes, or plant parts that may not be collected, seasonal restrictions and important safety practices related to collection [11]. Hence field observations and sample collections of those fields are restricted.

2.2 Lack of Appropriate Equipment to Support Required Conditions for the Pathogen

Maintaining an optimum temperature and safe moisture content for the pathogen during transport and avoiding damage during transportation is critical for the collected plant materials. Hence, without proper equipment for smooth transportation leads to the deterioration of the collected samples. For instance, plastic bags should be used for most plant samples, including

leaves, stems, and roots to prevent samples from drying out during transport. Nevertheless, in stages of decay, fleshy fruits, vegetables, or tubers should be wrapped individually in dry papers [12].

2.3 Different Perceptions

Diseased plants sometimes have been infected by one or more pathogenic microorganisms, and affected plants often display a mosaic of combined symptoms [12], leading misidentifications or constraints in culturing and identification. Different persons might judge the same phenomena differently. Only observers with technical knowledge about plant pathogens can make scientific observations and correctly identify the target plant pathogen [13]. E.g., colour variations and necrosis due to nutrient deficiency or viral infections may be misidentified as a fungal disease. Therefore, observations and recording details related to plant pathogens are highly skilled technical tasks and only well-trained people should be selected for these practices. In mass collections, insufficiently trained people to make required collections is problematic.

2.4 Infrequent Observation and Recording

Most plant pathogenic observations should be performed in the field. Recording the results of some experiments, such as pathogenicity tests and in vivo antagonistic activities, should be carried out in the fields. Therefore, frequent visits to the original collection sites are essential. If the original collection sites were barely accessible, the researchers could not access and record observations frequently. However, sometimes it may be restricted by distance to the site, constraints on labor, clearance of the host plants in the field by growers, funds availability, and etc.

2.5 Plant Quarantine Regulations

Plant pathogens especially microorganisms cause severe losses or damage to crops worldwide and significantly reduce the quality and quantity of agricultural commodities. These losses pose a major annual threat to global food production [14]. Plant quarantine is therefore designed to safeguard against harmful pathogens exotic to a country and restricts the movement of pathogens between geographical areas. In the case of overseas transfer (if the current country does not have sufficient facilities to conduct the required pathogenic investigation at the ports), it is not easy to have a smooth transfer because of these restrictions. Most of the time investigations are hindered by these quarantine issues. The import is prohibited when the risk of the pathogen is very high and the safeguards available in the country are inadequate [15,16].

3. LIMITATIONS IN MORPHOLOGICAL METHODS OF IDENTIFICATION

Out of the estimated 2.2–3.8 million species of fungi, less than 5% have been described. The remaining 95% are more tractable with molecular techniques than conventional methods, which only adds 1000 new species per year [7,17]. However fast and quick detection and identification of plant pathogens are essential to reduce the disease spread and facilitate effective management practices [7]. Thus, fast, accurate, reliable diagnostics in the field and correct identification of pathogens are significant for plant pathogen management.

In the pre-molecular era, the detection and identification of plant pathogenic fungi mostly depended on microscopic, morphological, and cultural approaches. Although the cornerstone of fungal diagnostics, these approaches can lead to unreliable results due to problems in accurate identification.

The conventional methods of identification include morphological methods in micro-fungi. The morphological structures of fungi include various types of spores, mycelium, and reproductive structures. These structures are varied based on the genetic makeup of the fungi, prevailing environmental conditions, substratum, light regimes, characteristics of the host, moisture content (relative humidity), availability, soil types and nutrient status of the soils and their availability,

pH and many other related soils and host factors where they were developed.

Conventional methods for identifying fungal plant pathogens relied on the interpretation of visual symptoms and/or the isolation, culturing, and laboratory identification of the pathogen. The accuracy and reliability of these methods depended largely on the experience and skill of the person making the diagnosis [18].

Systematics is the key to the identification of an organism. In the past, fungi and other organisms had been identified based on their morphological characteristics. The Saccardo system for asexual fungi was primarily based on the morphology of sporulation structures as they were known in nature as well as the morphology and pigmentation of conidia and conidiophores [19]. The morphological features used to identify fungi may vary from organism to organism but largely depend on their characteristics in culture under a standardized condition. For example, to have a good culture for identifications, certain media had to be used for sporulation, and specific diurnal temperature and lighting regimes need to be followed, especially for the *Fusarium* spp. [20].

Crous et al. [21] in their review on identifying and nomenclature of plant pathogenic fungi, gave a chronological and systematic assessment of conventional methods of identification. The first evidence of plant disease was reported in the 1840s which was caused by a plant pathogenic fungus *Phytophthora infestans* [22]. While in the early stage of the twentieth century, different fungal structures were given the emphasis on classification systems of fungal pathogens in potatoes. In the mid-nineteenth century, spore characters were accepted widely in classification [23]. After having the light microscope (LM), details of finer structures of fungi were revealed and were linked to differences in ontogeny and gradually accepted the earlier spore-based systems; families and genera started to be rearranged in the later part of the twentieth century. The appearance of scanning electron microscopy (SEM) in the mid-1960s helped examine the ornamentals of spores, helped in the separation of otherwise very similar plant pathogens, and clarified patterns of conidiogenesis [24]. Then came the era of Transmission electron microscopy (TEM), which led to the discovery of fundamental differences in the major groups [25].

During the 1960s and 1970s thin-layer chromatography (TLC) and isozyme profiles were used to find the chromosome numbers e.g., Wieloch [26]. Vegetative compatibility groups (VCGs) were developed, which was important in many research studies on pathogenic *Fusarium* spp. [27]. The cluster analysis was performed after having powerful computers in the 1970s, revealing large numbers of morphological, cultural, and physiological characteristics; similarities were then computed and analyzed. Since applying Nomarski differential interference contrast, the LM significantly improved in the 1980s, but separating identical fungi was still challenging.

Before developing the fungal disease in a crop, fungal spores must be released, dispersed, and deposited on the substrate; later, if environmental conditions are favorable, spores will germinate in the affected areas, forming infective structures [28]. The host plant should be susceptible to the infection for successful disease development and interactions with the pathogen (Figure 2). Temperature plays a key role in the germination of conidia and subsequent mycelia growth and thus influences both the onset and the severity of plant diseases. In many species of filamentous fungi, high temperatures (25°C) stimulate enzyme activity and promote the development of germ tubes and infective structures [29,30].

Along with the other factors which were important in the morphology of plant pathogenic microfungi, the temperature played a significant role in the germination of spores, infection, diseases development and finally, spore morphology (e.g. shape, size and colour) of plant pathogenic microfungi. The Ph.D. research studies (M.A.U. Mridha, personal communication) indicated that

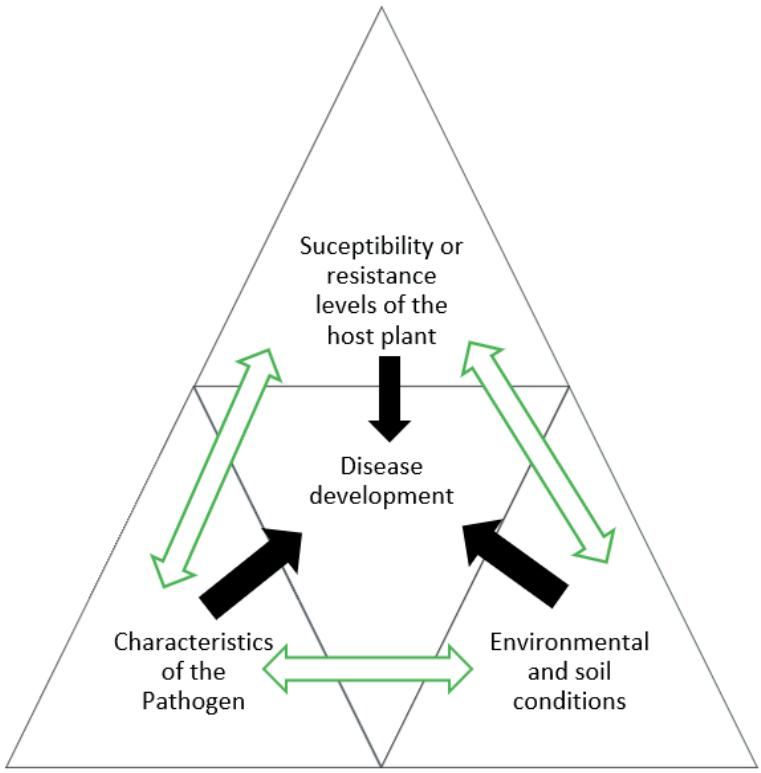


Figure 2. Disease triangle to depict the interaction among the host and pathogen with environmental and soil conditions.

Alternaria brassicae of winter oil seed rape did not produce any spores at 29ºC. Under laboratory conditions on PDA medium and filter paper methods, very few spores were produced at 5ºC and 10ºC; at 15ºC a high number of spores but abundant spores with good morphology at 20–25ºC. The moisture content also showed a substantial impact on sporulation, spore production and the morphology of the spores. For example, high moisture was preferred in developing spores of *A. brassicae*. The substratum, light regime and pH value also played significant roles in producing spores and the morphology of the spores. The results indicated that a high variability was observed in spore morphology because of the different factors studied. These types of variability may cause an important limitation in identifying plant pathogenic microfungi. A change in lighting regime, temperature, or multiple transfers can also affect the colour of an isolate [20], which may cause a problem to identify correctly by following the morphological characteristics of the fungi. Significant variability in morphology and pathogenicity was recognized among isolates from different host species and between isolates from different parts of the same plant [31], causing the problem of morphological identification.

Despite the widespread use of classical methods compared to sampling using molecular techniques for fungal identifications, there are limitations or certain disadvantages. Some species may not grow or produce reproductive structures in culture and these structures cannot often be seen in natural settings. These species will be

misidentified by traditional sampling methods, even though they could be important members of the plant pathogenic fungal community. The fact that some species will not be detected clearly has the potential to bias classical studies. It is difficult to assess how many species are missed by classical techniques or to determine if this can bias the results of any particular study. While molecular-based studies of fungal diversity can provide an independent assessment of the fungal community, they are limited to sampling a small area, which can result in a different set of biases. Classical sampling methods can be considerably more time-consuming than molecular techniques. Additionally, more taxonomic expertise is required for classical methods than molecular methods, as all species must be identified based on morphological characters. The relative scarcity of trained taxonomists can lengthen the time it takes to identify the collections and thereby lengthen the time it takes to carry out any study [32]. Further, it may lead to wrong identification by non-experts.

Therefore, it is necessary to fast-track the pace of species description to approach a complete catalogue of fungal diversity within a reasonable time frame. However, the disadvantage of traditional morphology-based taxonomy and the massive number of active taxonomists make it highly unlikely to achieve the goal soon [33]. The most common restrictions of traditional taxonomic analyses were limited taxonomic characters. Traditional biological information for classifying fungi into major groups included morphology, ultrastructure, physiology, tissue biochemistry, ecological traits [34] and chemotaxonomic traits [35]. Phylogenetic studies have demonstrated that many morphologically similar taxa might represent distinct lineages, and numerous well-known species were, in fact, species complexes [36].

Species differentiation was based on colony morphology and morphobiometric characteristics analysis, including conidia distribution on conidiophores, size, shape, colour, texture, and the number of septa. In all cases, observations were made on fresh preparations of young and mature areas of colonies [37,38,39].

Despite recent advances in molecular methods, classical methods have many advantages for studying fungal diversity. The advantage of classical methods is that they are generally less expensive and need less specialized equipment compared to molecular methods. These are important considerations for many investigators, especially those in developing nations [32].

4. Isolation-based limitations

It is usually necessary to isolate and culture fungi from the living or freshly collected specimens before they can be identified or used for further experiments. Above all, the aim of isolation is to obtain single colonies of the suspect pathogen, to obtain the pure cultures containing only one species. Facultative parasites or necrotrophs capable of saprobic growth can generally be isolated and grown in culture, although some of these may vary based on their requirements. Isolation of fungi from plant material is usually achieved either by single or multi-spore methods, which may not be possible with pathogenic microfungi or other endophytic pathogens most of the time [40].

Once returned from a collecting trip, the specimen may need sorting or categorizing based on its freshness. Sample deterioration is the first constraint in the isolation of plant pathogenic fungi. Some specimens will deteriorate quickly (e.g., larger fleshy macrofungi including many Basidiomycetes and some Dothideomycetes, Pyrenomycetes and Sordariomycetes). These specimens need quick isolation right after returning from the collecting trips. Since the pathogenic fungi colonizing the inner tissues must be isolated, it may also not be possible to dry them in a dryer or a plant press or freeze them at lower temperatures for several days until examination [28]. Thus, the isolation of these pathogenic fungi is often challenging.

The choice of specimens for isolating plant pathogenic fungi requires care. The best plant

samples to collect for isolation are those in the early to middle stages of the disease development, where the pathogen is still in the active growth phase. Severely diseased plant samples are often useless during isolation because the pathogen may no longer be viable [41]. Many saprobic fungi grow on and contaminate plant tissue as secondary colonizers of disease lesions. These saprobic fungi may outgrow and mask the primary pathogen, making the isolation of the pathogen difficult. Therefore, at the isolation, if the specimens are not at the appropriate disease developmental stage, it may result in isolating only the saprobes, and the pathogen isolation is failed. The choice of material plays an important role in successfully isolating pathogenic fungi. In some cases, the symptoms may appear in one part of the plant, but the pathogen is found elsewhere; for example, wilt disease symptoms appear in the leaves, although the pathogen occurs in the vascular system of the roots and stems [28]. Therefore, a basic knowledge of symptoms and their developmental patterns will be helpful to ensure that the plant part collected is infected by the pathogen.

Knowing symptoms is important to determine whether the disease is abiotic or biotic. Usually, the plants exhibit similar symptoms when there are abiotic diseases, which are caused by conditions external to the plant, not living agents (e.g., nutritional deficiencies, soil compaction, salt injury, ice, and sun scorch). They are not infectious but are very common and should be considered when assessing the health of any plant.

There are different techniques available for the isolation of plant pathogenic fungi. However, the exact procedure for isolating fungal pathogens should be decided upon the nature of the host plant material and the pathogen itself, and it would be the accurate decision of an expert. Lack of knowledge and attention on these specific techniques will cause failures in isolating the pathogenic fungi. For example, if the plant part used for isolation is a symptomatic leaf, you have to choose leaves with young lesions because the fungus will be at its most active phase. However, suppose the plant part is a stem with deep lesions. In that case, samples must be taken from internal tissues to avoid the need for surface sterilization, and the sample should be split/dissected longitudinally from the healthy to the diseased area [28].

One frequently faced constraint in isolating plant pathogenic fungi is that sometimes disease symptoms are apparent, but the causal pathogen cannot isolate. Despite the technique and the medium used, the isolation attempts may fail. In such cases, the common practice is to incubate the plant material in a moist chamber to induce the formation of fruiting bodies and sporulation. However, with the incubation, the growth of saprobes occurring on the plant surface is also encouraged. Even though a quick surface cleanup with ethanol or low-concentrated surface cleaning agents like sodium hypochlorite may be helpful, it may also damage the structures of the pathogen. Usually, there are plant pathogens that are host specific, and during the isolation, their requirements must be provided in vitro to culture them [42]. For this purpose, the symptoms of the plant parts with the disease must be observed carefully, and referring to the previous literature would be worth determining the host specificity of the potential pathogens. Then selective media can be used or if not, extracts from the host tissues can be added to the medium prior to the isolation.

Isolation of root pathogens and soil-borne pathogens is rather different as well as challenging from those of foliar pathogens [43]. Root pathogens colonize the rhizosphere, and together with these harmful fungi, there are also beneficial mycorrhizal fungi [44]. Nevertheless, soil-borne pathogens may not evenly disperse in the soil. Instead, they tend to aggregate at favorable conditions (e.g., high moisture content, high nutrient availability) or else surrounding the infected parts. Also, during the collection, mechanical damage may occur while using soil bore-like equipment, and this will disrupt the hyphae and mycelia can be separated [45].

Therefore, isolation of root pathogens involves additional steps like excessive washings and serial dilutions, as well as several random sampling for an accurate assessment, should be done.

Isolating plant pathogenic fungi became much more difficult when plants were asymptomatic during visual inspection or field collection [46]. They may be latent pathogens that are quiescent in the early maturity stage of the plant and may transfer themselves into other parts of the plant later on to develop diseases. Due to this reason, many pathogenic fungi may go undetected and undiscovered at the initial stages. Samples may not be available for isolation as they are without symptoms [47]. This will increase their establishment in natural and semi-natural ecosystems [48].

5. CULTURE-BASED LIMITATIONS OF IDENTIFYING PLANT PATHOGENIC MICRO-FUNGI

Improving the ability of rapid plant pathogenic micro-fungal identification and diagnosis will significantly increase the possibility of reaching containment of pathogens [49]. Pathogenic taxa are traditionally described based on morphological and phenotypic characteristics [50]. To date, DNA-based phylogenetic methods have been applied to complement since closely related species may be morphologically indistinguishable through traditional culture methods [49]. Traits within a species can also vary depending on culture conditions [51]. Hence, these problems often make the accurate identification of plant pathogens a challenge. Furthermore, it is important to archive pathogen cultures and related data support for pathogen recognition and diagnosis [49].

An expert can recognize some diseases by observing the symptoms or signs on infected tissues (e.g., mildew on leaves, fruitbodies). There are many diseases and symptoms that cannot be differentiated visually from one another, causing complications in diagnosing the pathogen. Thus, additional procedures for detection are needed to spot the cause of the disease [52]. Isolation of the fungi and morphological or/and molecular identification of the pathogen are necessary as specific microorganism is unknown in many cases [53]. To isolate fungal pathogens from plants, a small slice of diseased tissue is placed on growing media [52]. Although the use of selective media, the isolation of pathogenic fungi is sometimes difficult due to the mass of unwanted and antagonistic fungi or bacteria, which rapidly overgrow the pathogenic fungi on the isolation plate [54]. Morphological and molecular characteristics can be used to identify pathogens from pure cultures. First, fruitbodies of the fungus (conidia and spores) are analyzed using light microscopy. However, this traditional pathogen identification method is time-consuming and requires skilled operators [55]. Therefore, the identification of fungal mycelium through molecular markers is introduced after DNA isolation from pure cultures [52].

To recognize the plant pathogenic fungi, PCR-based methods have been used frequently. These methods could effectively recognize the pathogenic fungi from cultured species and uncultured taxa from the natural environment [56]. Culture-based identification is more reliable than uncultured one. However, if we cannot provide pure culture from our isolation, it will not be helpful in correct identification [57,58]. There are several limitations in culture-based identification, such as suitable culture medium and storage conditions [49].

Some fungal cultures are negative on bacterial media [59]. Malt extract agar (MEA), potato dextrose agar (PDA), dilute potato dextrose agar (DPDA), and water agar (WA) are the commonly used media for fungal culture [60]. Chloramphenicol or any antibiotics is used to prevent the bacteria contamination of the medium, but it will reduce the yield of many opportunistic fungi [61]. All plant pathogenic fungi do not grow in the same culture media; therefore, the culture medium suitable for the selected fungi should be confirmed or checked. However, without having relevant references, it will time consuming process. Without a suitable

culture, this method could not be used in plant pathogenic fungi identification [62].

Cultures should be incubated in a moistened environment for 21 days at 15-37 ºC because it is the average temperature range for the incubation of fungi [63]. This incubation temperature will change according to the specimens where we collected fungal species and their natural environmental condition. They should be inspected daily for at least a week and at least three times weekly after that. Some fungi need more prolonged incubation as they grow very slowly [64].

Once colonies are visible, they should be scrutinized for their morphological characteristics, the color of the colony, and the asexual or sexual structures formed on the colony. Other than morphology, these pure cultures can be used to extract DNA for further molecular identification [65]. The major limitation of this culture technique is that it could not obtain cultures for all pathogenic fungi and pure cultures without contamination.

6. LIMITATIONS OF APPLYING KOCH'S POSTULATES

Koch's postulates are significant in establishing the criteria that a specific microorganism causes plant disease. Koch's postulates can be summarized as follows [66];

• The same organism must be present in every case of the disease.

• The organism must be isolated from the diseased host and grown in pure culture.

• The isolate must cause the disease when inoculated into a healthy, susceptible host.

• The organism must be reisolated from the inoculated, diseased host.

Even though these stringent criteria provided a framework for thinking about the proof of microbial disease causation, scientists uncovered many exceptions to Koch's postulates during the next decade. Due to the numerous limitations of the postulates, it was evident that they could not be applied to all microorganisms. According to Koch, for a microorganism to be considered the causative agent, it must have met the above-described conditions. Therefore, from time to time, revisions and additions were published related to Koch's postulates to address these limitations [67]. According to the first part of Koch's postulates, the pathogen must always be isolated from the host of the disease and grown in pure culture. In this situation may not always be possible to establish if the pathogen is difficult to culture or if its occurrence preceded the development of the symptoms. In such a situation, the pathogen may not be able to grow in an artificial medium; hence it will not be compliant with Koch's second postulate, which requires the organism to be grown in pure culture. Though they cannot fulfill Koch's postulates, they are unequivocally pathogenic [68]. According to the second part of Koch's postulates, the pathogen should be inoculated, and once the healthy host is inoculated, it should develop the disease symptoms. However, not all hosts exposed to a pathogen will acquire the infection. Whether the plant is infected or not may depend upon the host and environmental factors. A plant's general health, proper functioning of plant physiology, acquired immunity from previous exposure to the same pathogen and plant's genetic variability will influence the infectious ability of the pathogen. Therefore, even the same pathogen may not be able to cause the disease again after the inoculation [69]. When there are situations where the same pathogen causes different diseases under different circumstances, complications may arise when following Koch's postulates. For example, *Puccinia* is a common pathogenic genus causing both leaf spots and leaf rust [70]. Plants may develop certain diseases from a single causative agent when the plant is under stress or based on environmental factors. At these times, symptom development may be contradictory to the originally observed symptoms, causing limitations in applying Koch's postulates [69,67].

In various situations, the host plant shows specific symptoms associated only with a particular pathogen. For example, *Phytophthora infestans*, the causative agent of the late blight in potatoes

develop distinctive pale green spots, *Fusarium* wilt by *Fusarium oxysporum* [70], that no other microbe can produce. There are plant diseases that may be caused by a community of microorganisms rather than a single pathogen. For instance, Leaf blight in carrot cultivars can be caused by both *Alternaria dauci* and *Cercospora carotae* [70]. These diseases usually result from a succession of pathogenic fungi occurring in different plant tissues. However, there are cases wherein different fungal pathogens show similar signs and symptoms that you cannot identify what specific pathogen causes the disease. Another major constraint in applying Koch's postulates is the host specificity. Diseased samples can be brought or sent to laboratories from different parts of the world. Once the causative agents are isolated and obtained cultures, they must be reinoculated into the host plant again to confirm the disease occurrence according to Koch's postulates. However, there can be instances where this may not be possible, particularly when the samples are sent from one part of the world to another part of the world (i.e., from temperate countries to tropical countries or vice versa). In such situations, getting the host plants for reinoculation is problematic.

Further, plants can develop silent diseases, especially during their early development. These pathogens, known as latent pathogens, remain quiescent within the plant; hence the plant is asymptomatic. A harmless fungus may cause disease later at the maturity stage of the plant when it can acquire extra virulence factors making it pathogenic. Certain pathogens can cause diseases in deep tissues by gaining access, i. e. vascular tissues of the plant. The symptoms may not be visible at the plant surface but produce an asymptomatic infection. Alternatively, only when the plant is under stress or harmed by pest attacks may these latent pathogens stimulate the disease development activating their pathogenic role. However, the absence of symptoms failed to fulfill Koch's postulates [69].

7. Limitations in phylogeny-based identification

Species demarcation using morphology and DNA-based approaches for closely associated taxa have been confirmed beneficial and incorporated for a variety of fungi, especially in studies of plant pathogenic fungi [71]. However, there are still remarkable limitations for plant pathologists when they identify fungi through DNA-based phylogeny methods. Especially where the fungi are to be associated with species complexes, plant pathologists cannot rely only on morphological approaches. The combination of DNA sequences of different gene regions allows a prevailing and frequent tactic for recognizing evolutionary heredities in fungi. Hence, precise identification of plant pathogenic fungi is essential for setting up quarantine procedures. Currently, there are remarkable incongruities amongst mycologists and pathologists when demarcating species boundaries within species complexes as the number of putative species ranges from few to more [72].

Species stay as basic units when designed for studies in ecological or evolutionary aspects. Hence, imprecise demarcation of species may allow inaccuracies in investigations that practice species as the base entity (e.g., phylogenetic public assembly evaluations). Ever since the first half of 90s, mycologists were regularly engaged with applying chromosome sequence data to calculate gene trees and species demarcation. The Genealogical Concordance Phylogenetic Species Recognition (GCPSR) [73] has been confirmed to be a better technique for species identification in fungi [74-76].

The significance of distinguishing cryptic/hidden species of plant pathogenic fungi has been extensively emphasized, and those kinds of research have improved rapidly over the earlier decades [77-80]. This was driven mainly by the growing accessibility of DNA sequences through the utility of phylogenetic investigations established on single or multi-locus sequence data. Diverse

populations could be discrete into different clades once incorporating tree reconstruction approaches; meanwhile, this is the prevailing indication in the data. Nevertheless, this may not be the only signal aiding species identification. On the other hand, a gene tree is not necessarily corresponding to the reality of a species tree, which represents the true relationship among species. For example, the high intraspecific disparity in ITS sequences was identified inside the *Neofusicoccum parvum-Neofusicoccum ribis* complex, and species previously described on that basis were revealed to be ITS haplotypes [81-84).

Granting the concatenation of multi-locus DNA sequences is influential and appropriate in calculating phylogenetic trees; these trees might not correspond with the species trees [85-87]. Hence, scientists have recently incorporated approaches based on the coalescent theory [85,88,89], which can make measurable estimates about the possibilities of gene trees and assist as a model for exploring reasons for gene tree discordance such as imperfect ancestry categorization, horizontal gene transmission, gene repetition and loss, hybridization, and recombination [88]. These techniques might elucidate random cut-offs [90] and over-supporting poorly determined clades [91]. Cruywagen et al. [92] estimated that many clades within *Lasiodiplodia* species in the concatenated gene trees were hybrids; some were well-supported and described as different species. Nevertheless, the outcomes of employing coalescent techniques were exclusively divergent. In other words, coalescent-based analyses did not sustain over-estimated species in the genus *Lasiodiplodia* acquired in concatenated multi-locus analyses.

Additionally, the general methodology of concatenating sequence data from multiple loci can also direct to deprived species discernment [93]. This highlights the significance of polyphasic methodologies when introducing new species in morphologically preserved genera of plant pathogenic fungi.

8. Suggestions to overcome limitations of identifying plant pathogenic fungi

As we discussed above, prior permission must be obtained from the relevant authorities before getting into the sample collection, especially when collecting the samples from national forests, crop fields, and private lands. There should be a simple and fast method to get permission from the relevant authorities and send fungal cultures abroad whenever necessary for identification purposes. Sometimes you may need to provide them with the collecting plan and inform them about the sampling materials and area. One or more pathogens can usually infect diseased plants; they may also have an abiotic disease that does not involve a plant pathogen. Affected plants often display several symptoms or visual signs of the infection [12]. Generally, each symptom of a particular disease would not appear on one plant within a diseased crop, and more than one plant organ can be affected by a particular disease. Therefore, it would be recommended to examine all the major plant organs for disease symptoms viz. roots, stems, leaves, and blossoms and to collect samples to include various plant organs. Additionally, several plant samples showing the range of symptoms may be necessary, as a single plant sample may not be enough to identify the causative agent correctly. Choosing samples with different stages of disease development (early and late) is required; adequate amounts are also equally important. However, dead materials are useless in disease diagnosis as various decomposing fungi and bacteria have invaded them, and the actual pathogens may no longer be extractable. Therefore, it is worth collecting living tissues in the early stages of the disease or in the process of dying and not already dead [12]. When the entire plant cannot be sampled it is better to collect the largest plant sample possible or portions of each major plant organ (e.g. roots, stems, leaves, flowers).

Suitable equipment should be prepared or assembled before sample collection, and adequately

trained personnel should be acquired before the sampling starts. Some preliminary required equipment must be specified and available are sample preservation equipment, sample containers and packaging materials, record-keeping devices, cameras, sample location markers, site maps, and Global Positioning System (GPS) recorders. In the insufficiency of trained people, a proper guide and training should be provided to those lacking sufficient field experience. Collected samples should not exposed to direct sunlight, keep them cool and do not allow them to dry out. Place samples in plastic bags in the shade or a cooler until they enter the laboratory. Leaves may be pressed between the pages of a book or magazine or wrapped in tissue. If entire plants cannot be sampled, photographs of affected plants can be taken. If possible, the research should be conducted in a nearby area that can regularly visit to perform Koch's postulates. Also, optimum growth may be obtained under more or less the same climatic conditions, and so on. If it is impossible, the researcher should use other alternatives, such as constructing greenhouses or high tunnels to continue the experiment. Further, if possible, the research should be done in the same country or region where the samples were made. If not, prior permission must be obtained from the relevant authorities for a proper quarantine clearance.

All data collected in the field should be sufficiently documented to avoid insufficient observation, recording and misidentifications. The documents used for this purpose should preliminarily include the following information: names of field sampling personnel, date and time of sampling, sample collection plan, sample locations, and physical and meteorological conditions [13]. Good information contributes to a better understanding of the problem. The sample should accompany a complete description of the problem and the crop's history. Provide the name of the plant submitted. Indicate when the problem appeared and when the sample was taken. Specify all fertilizers and pesticides used. Examine the growing site carefully and note the conditions. Make a note of environmental conditions for the site, such as elevation, flooding, previous crop history. Indicate any observable pattern of disease occurrence (for example, in random patches or uniformly throughout the crop). This information is helpful in making a distinction between damage caused by pests and damage caused by other factors.

9. Conclusions

The requirement to classify fungus swiftly and precisely is constant. Rapid and precise identification of fungal infections to species or strain level is frequently necessary for disease surveillance and the implementation of a disease management strategy. Challenges associated with the identification of plant fungal pathogens can be overcome by proper precautionary actions, using appropriate sample collection techniques with the proper field, and morphological observations. Fungal isolation, obtaining pure cultures, and applying Koch's postulates in pathogen confirmation are very important steps leading to accurate pathogen identification. Although the exact number of fungal species is unknown, environmental DNA sequencing could increase the precision of current estimates. Polyphasic methodology and morphological comparisons are crucial in identifying plant fungal pathogens especially in confirming species novelties. Additionally, next-generation sequencing will offer intriguing methods for extending the scope of molecular-detection studies.

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Conflict of interest statement

The authors declare no conflict of interest.

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