Vol. 14, No. 3, pp. 674-684, August 2026 DOI: https://doi.org/10.23960/jat.v14i3.10637

Jurnal Agrotek Tropika

P-ISSN: 2337-4993
E-ISSN: 2620-3138

Journal homepage: https://jurnal.fp.unila.ac.id/index.php/JA

GROWING RESOURCE MODIFICATION EASES THE COMPLICATIONS IN ISOLATING Mycosphaerella ORIGINATING FROM BANANA

MODIFIKASI KONDISI PERTUMBUHAN DALAM MENGATASI KERUMITAN ISOLASI Mycosphaerella ASAL PISANG

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ARTICLE HISTORY:

Received: 16 April 2025
Peer Review: 04 June 2025
Accepted: 22 July 2026

KEYWORDS:

Amoxicillin, oatmeal agar, photoperiod, pH, young infected leaves

ABSTRACT

Experimental study on the Mycosphaerella species complex was often limited by the availability of the isolates due to problems in the isolation process. Two symptomatic leaves from initial and later infections were used as the source of isolates. As critical environmental factors for fungal development, we observe different pH (5.5, 6.0 6.5, and 7.0) and antibiotics (chloramphenicol and amoxicillin) that are suitable to give less-contamination growing resources for Mycosphaerella. Subsequently, we analyze the three different media, i.e. potato dextrose agar, oatmeal agar, and V8 juice agar for the optimal growth of Mycosphaerella under varied photoperiod for incubation (12/12 light/dark cycle and 24-h dark) using the selected pH and antibiotics. This research was conducted in an ambient laboratory setting utilizing a complete randomized factorial design with ANOVA and Tukey test. The subculturing stage of Mycosphaerella was shown to be most successful at pH 6.0 with amoxicillin from a young infected leaf with first signs (brown streak without halo). This fungus preferred oatmeal agar with 24-hour dark incubation to achieve the highest mycelial growth (1.6 mm/day) and conidial production (3.3 x 10^4 conidia ml^{-1}). This could be an essential approach employed by researchers to provide a sufficient quantity of Mycosphaerella propagules in vitro, in vivo, and ad planta trials for Sigatoka disease management.

KATA KUNCI:

Agar-agar oatmeal, amoksisilin, gejala awal infeksi, periode cahaya gelap, pH

ABSTRAK

Kerumitan isolasi kompleks spesies Mycosphaerella seringkali menjadi faktor pembatas penelitian yang komprehensif mengenai patogen penyebab penyakit Sigatoka pisang. Daun pisang terinfeksi pada gejala awal dan gejala lanjut digunakan sebagai sumber inokulum untuk mengisolasi cendawan ini. Sebagai tahap seleksi awal, beberapa perlakuan pH (5.5, 6.0, 6.5, dan 7.0) serta dua jenis antibiotik (kloramfenikol dan amoksisilin) diuji untuk memperoleh kondisi lingkungan yang kritis bagi pertumbuhan Mycosphaerella dengan kontaminasi yang minim dari cendawan saprofit lain. Selanjutnya, dilakukan pengamatan pertumbuhan cendawan ini pada tiga jenis media yaitu potato dextrose agar, agar-agar oatmeal, dan V8 juice agar pada pH dan antibiotik yang telah terseleksi pada pengujian sebelumnya. Seluruh tahapan penelitian ini dilaksanakan pada kondisi laboratorium menggunakan rancangan faktorial acak lengkap dan dianalisis ragam dengan uji lanjut Tukey. Hasil penelitian menunjukkan bahwa daun terinfeksi dengan gejala awal (bercak coklat bergaris yang belum dikelilingi halo) merupakan sumber inokulum terbaik dan keberhasilan tertinggi untuk isolasinya diperoleh pada media ber-pH 6 yang ditambahkan antibiotik amoksisilin. Di antara tiga media yang diuji, cendawan ini tumbuh optimal pada media oatmeal agar pada kondisi inkubasi 24 jam gelap, yang menghasilkan pertumbuhan miselium yang baik dengan laju pertumbuhan 1.6 mm per hari dan pembentukan konidia mencapai 3.3 x 10^4 konidia ml^{-1}. Pertumbuhan Mycosphaerella pada titik-titik kritis tersebut di atas dapat digunakan dalam penyediaan inokulum cendawan ini untuk pengujian in vitro, in vivo, dan in planta dalam rangkaian pengendalian terpadu penyakit Sigatoka pisang.

1. INTRODUCTION

Banana productivity is frequently constrained by fungal diseases. In addition to *Fusarium oxysporum* f. sp. *cubense*, the causal agent of Fusarium wilt and one of the most destructive diseases affecting banana production worldwide, the *Mycosphaerella* species complex (anamorph: *Pseudocercospora*), which causes Sigatoka leaf spot disease, is another major fungal pathogen responsible for global yield losses exceeding 65% (Shantiyaa *et al*., 2013). Integrated disease management remains the primary strategy for suppressing Sigatoka disease and typically combines field sanitation, biological control agents, and synthetic fungicides. Fungicides containing trifloxystrobin, either applied alone or in combination with azole fungicides, have been reported to effectively suppress Sigatoka disease by inhibiting fungal sporulation (Essis *et al*., 2023; Nagesh *et al*., 2023). These management strategies primarily target the reduction of both sexual spores (ascospores) and asexual spores (conidia), which serve as the primary and secondary infectious propagules in the Sigatoka disease cycle.

The genus *Mycosphaerella* (phylum Ascomycota) comprises more than 10,000 described species, and its taxonomy has undergone extensive revision over the past decades (Crous *et al.,* 2000). To improve the accuracy of species identification, various molecular approaches have been developed. Arzanlou *et al*. (2007) developed a TaqMan real-time PCR assay targeting the β-*tubulin* gene for the detection and quantification of the three *Mycosphaerella* species associated with Sigatoka disease. In addition, molecular characterization of *Mycosphaerella* spp. has been widely conducted using the internal transcribed spacer (ITS) region, together with *actin*-based markers specific to the genus. Species-specific primers targeting the *actin* gene have also been developed to distinguish the three major Sigatoka pathogens infecting banana, namely MFact for *M. fijiensis*, MMact for *M. musicola*, and MEact for *M. eumusae*. Furthermore, mating type (Mt)-specific primers for *M. fijiensis* were developed using single-conidium isolates obtained directly from symptomatic banana leaves (Conde-Ferráez *et al*., 2010; Vázquez-Euán *et al*., 2012).

Despite substantial advances in the molecular characterization of the *Mycosphaerella* species complex, the production of fungal inoculum remains a major challenge. Thomas and Saranavakumar (2019) reported that one of the principal difficulties in working with fungi belonging to the *Cercospora–Pseudocercospora–Mycosphaerella* complex is their slow growth and poor sporulation on artificial media. Consequently, the selection of appropriate culture media and incubation conditions is critical for the successful isolation, growth, and sporulation of *Mycosphaerella* under laboratory conditions.

Fungal growth is strongly influenced by its growing environment. Critical factors, including nutrient composition, light regime, temperature, water availability, and medium pH, can markedly affect the development of both somatic (vegetative) and reproductive fungal structures (Alberctsen & Witzell, 2012; Menge, 2023). Zhang *et al*. (2015) successfully cultured *Mycosphaerella* isolated from pea on potato dextrose agar (PDA) under a 14-h fluorescent light photoperiod at 20 °C. Similarly, Kumakech and Opio (2023) evaluated six culture media at different pH levels for *M. fijiensis* isolated from banana and reported that malt extract agar (MEA) adjusted to pH 5.5 provided optimal conditions for amino acid and nitrogen availability, thereby promoting fungal growth. Although these studies provide valuable references, their findings may not be directly applicable to *Mycosphaerella* isolates originating from Indonesia because different *Mycosphaerella* species and populations may exhibit considerable physiological variation and adaptation to local agroecosystems. Therefore, studies evaluating the colony characteristics and sporulation of Indonesian *Mycosphaerella* isolates are required to establish optimal culture conditions for inoculum production. Accordingly, this study aimed to optimize culture conditions for inoculum production of the *Mycosphaerella* species complex.

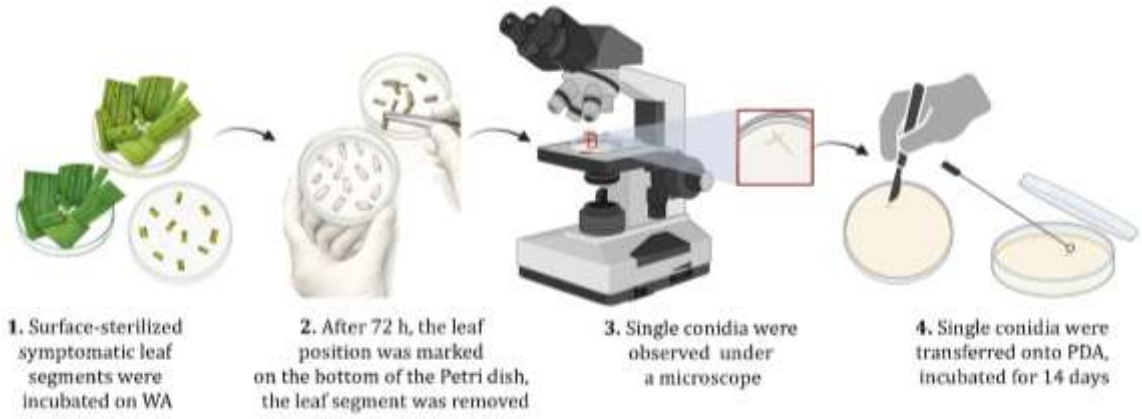

Figure 1. Isolation procedure of *Mycosphaerella* sp. from symptomatic banana leaves. (The schematic illustration was created using BioRender)

2. MATERIALS AND METHOD

2.1 Isolation of *Mycosphaerella* sp.

Symptomatic Cavendish banana leaves serving as the inoculum source were collected from the Cikabayan Experimental Farm, Faculty of Agriculture, IPB University, West Java (-6.55368393, 106.72368918). Leaf samples representing two stages of streak symptom development: (1) early streak lesions without a chlorotic halo and (2) advanced coalescing streak lesions with a chlorotic halo, were incubated on water agar (WA) for 48–72 h. At 72 h after incubation, the emergence of single conidia was examined under a stereo microscope and confirmed under a compound microscope at 40× magnification. Individual conidia were aseptically transferred using a sterile scalpel onto potato dextrose agar (PDA) plates and incubated for 14 days (Figure 1). The isolates were identified based on their macroscopic and microscopic morphological characteristics. Those consistent with the characteristics of *Mycosphaerella* spp. were selected for subsequent experiments.

2.2. Optimization of pH and antibiotic conditions for *Mycosphaerella* spp.

Fourteen-day-old *Mycosphaerella* colonies were used to evaluate the effects of medium pH and antibiotics on fungal isolation. Mycelial plugs were excised from actively growing colonies using a sterile cork borer and placed at the center of potato dextrose agar (PDA) plates. The pH of the PDA medium was adjusted to four levels (5.5, 6.0, 6.5, and 7.0) using NaOH or HCl, with 10 replicates per treatment. At each pH level, the medium was supplemented with either chloramphenicol or amoxicillin. The effectiveness of each treatment was evaluated based on the isolation success rate of *Mycosphaerella* spp. and the ability to minimize the occurrence of non-target fungi.

2.3 Optimization of growth medium and lighting conditions for *Mycosphaerella* spp.

The pH and antibiotic combination selected from the previous experiment was used for further optimization of the culture conditions for *Mycosphaerella* spp. Three culture media, potato dextrose agar (PDA), oatmeal agar (OA), and V8 juice agar (V8A), were evaluated. Two lighting regimes, a 12 h light/12 h dark photoperiod and 24 h continuous darkness, were tested to assess the growth of *Mycosphaerella* spp. The isolates were incubated at room temperature (25 ± 2 °C), and colony diameter was measured at 7, 14, and 21 days after incubation (DAI).

The same culture media and lighting regimes were also evaluated to produce ascospores and conidia. Agar blocks (1 cm^2) containing fungal colonies were placed on glass slides and incubated in

Petri dishes. Conidial production was assessed at 5 DAI. The microscopic characteristics of *Mycosphaerella* sp. were examined using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) at 400× and 1000× magnification.

2.4 Experimental Design and Data Analysis

The experiment evaluating culture media was arranged in a 3 × 2 factorial completely randomized design (CRD), consisting of three culture media and two lighting conditions, with 10 replicates per treatment. Colony growth was assessed three times at 7-day intervals, beginning at 7 days after inoculation (DAI). The same experimental design was used for the conidial production assay, with three replicates per treatment, and observations were conducted daily from 1 to 7 DAI. Data were analyzed by analysis of variance (ANOVA) using SAS version 9.1. When significant differences were detected, means were separated using Tukey's HSD test at $P < 0.05$.

3. RESULTS AND DISCUSSIONS

Sigatoka disease caused by the *Mycosphaerella* species complex is characterized by dark brown to black lesions on the upper leaf surface, with lesion shape, color, and size varying among species. Advanced symptoms are more readily recognized, as individual lesions enlarge and coalesce into blight-like, diamond-shaped, or elongated elliptical necrotic lesions surrounded by chlorotic halos. However, at this advanced stage, banana leaves are frequently colonized by other fungi, particularly *Neocordana musae*, making it more difficult to distinguish the original symptoms caused by the *Mycosphaerella* species complex. Therefore, accurate recognition of the characteristic early symptoms is critical for the successful isolation of *Mycosphaerella* spp., which are known to be difficult to isolate.

Banana leaves exhibiting streak lesions, the characteristic symptom of *Mycosphaerella* spp. infection, were selected as the inoculum source in this study. Isolations were performed from both early-stage streak lesions and advanced lesions (Figure 2a, b). The isolation results showed that *Mycosphaerella* was recovered more successfully from early-stage streak lesions, representing the initial phase of the primary infection cycle (Figure 2a). These early lesions provided the most suitable inoculum source because they had not yet been extensively colonized by other parasitic or saprophytic fungi, which typically grow rapidly on artificial media and interfere with fungal isolation. The absence of a chlorotic halo around the streak lesions also indicated that the symptomatic tissue was still predominantly colonized by the primary pathogen, which was actively growing and progressing through its infection cycle.

The present study showed that banana leaves exhibiting early streak symptoms were the most suitable inoculum source for isolating *Mycosphaerella* spp. Gomes *et al*. (2013) also used leaves representing different stages of symptom development (specks, streaks, and spots) for the isolation of *Mycosphaerella* spp. and reported that conidial production was highest at the spot stage. However, they did not compare isolation success among the different symptom stages. Monosporic cultures of *M. fijiensis* have also been established from single ascospores released from pseudothecia on necrotic lesions (Manzo-Sánchez *et al*., 2019). Unlike the present study, they did not investigate whether the stage of symptom development influenced the isolation success. Thus, the present study provides new evidence that early streak lesions are the most suitable inoculum source for obtaining pure isolates of *Mycosphaerella* spp.

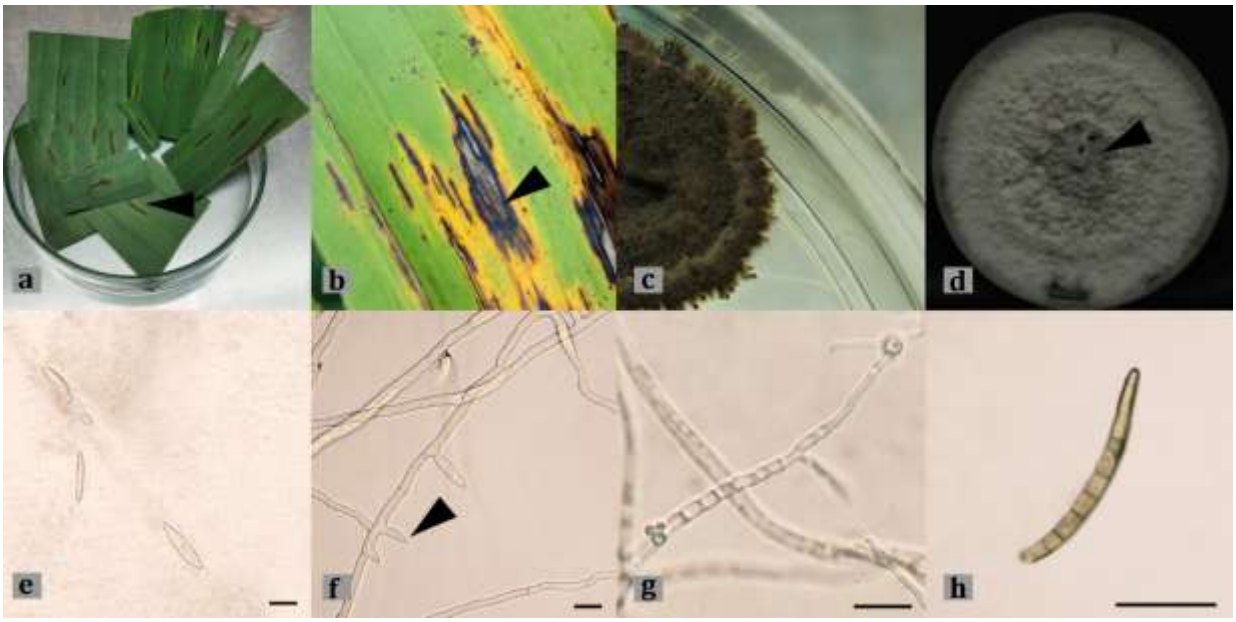

Figure 2. Symptoms and morphological characteristics of the pathogen causing Sigatoka leaf spot in banana: (a) early symptoms, (b) advanced symptoms, (c) colony morphology of the primary isolate at 7 DAI, (d) subcultured colony on oatmeal agar supplemented with amoxicillin, showing stromatal masses in the oldest region of the colony at 35 DAI, (e) ascospores, (f) young conidia produced sympodially at the tips of conidiophores, (g) late stage of conidiogenesis, and (h) mature conidia. Scale bar = 10 μm.

Mycosphaerella colonies exhibited slow growth. Colonies obtained directly from infected leaf tissues adhered firmly to the surface of PDA medium and were characterized by a rough texture and dark pigmentation (Figure 2c). In contrast, subcultured colonies grown on PDA at room temperature developed a smoother colony surface with light gray pigmentation, and pseudothecia were observed in 18-day-old cultures (Figure 2d). Such phenotypic variation between primary isolates and subcultures is commonly observed in fungi and may be associated with differences in growth conditions, nutrient availability, and the differential expression of genes involved in secondary metabolism. Microscopically, the fungus was characterized by asci containing eight ascospores. The ascospores were diamond-shaped, with apices that were acute, rounded, or slightly notched (Figure 2e). Conidiophores developed as modified hyphae and produced conidia sympodially at their apices (Figure 2f). Conidiogenesis occurred concomitantly with septum formation, and mature conidia were 5–6-septate, with darkly pigmented walls and hila (Figure 2g–h). These colony and microscopic characteristics are consistent with the descriptions of *Mycosphaerella* species reported by Crous *et al.* (2011).

Mycosphaerella musicola, *M. fijiensis*, and *M. eumusae* have been reported as the causal agents of yellow Sigatoka, black Sigatoka, and eumusae leaf spot (formerly known as Septoria leaf spot) of banana, respectively. These diseases are characterized by symptom development from small lesions to irregular dark brown to black necrotic spots (Carlier *et al*., 2000; George *et al*., 2022; Kema, 2024). In local banana cultivars from South Kalimantan, Indonesia, the *Mycosphaerella* species complex causes disease severity ranging from 15% to 70%. In the same study, several local banana cultivars exhibiting resistance to the pathogen were identified, with an incubation period approximately four times longer than that of susceptible cultivars (Mariana *et al*., 2017).

During the isolation period, the predominant saprophytic fungi recovered from the leaf tissues belonged to the genus *Penicillium*, followed by *Helminthosporium*, *Cladosporium*, and *Aspergillus*. The dominance of *Penicillium* is not unexpected because this genus exhibits a broad tolerance to environmental conditions. Van Long *et al*. (2017) reported that *Penicillium* spp. can grow over a wide

pH range (2.0–8.0), and although conidial germination declines substantially at low temperatures, germination can still occur at temperatures as low as 5 °C. These characteristics enable *Penicillium* spp. to readily colonize plant tissues and compete with more slowly growing fungi during isolation. Therefore, optimization of both medium pH and antibiotic supplementation is essential to improve the selective isolation of *Mycosphaerella* on PDA. The present study demonstrated that isolation success was primarily influenced by medium pH, followed by the type of antibiotic used (Table 1).

As shown in Table 1, the choice of antibiotic supported the successful isolation of *Mycosphaerella*, with medium pH serving as the critical factor. In the presence of amoxicillin, at least 20% of *Mycosphaerella* colonies were recovered across the pH range of 5.5–7.0, whereas no *Mycosphaerella* colonies were recovered on chloramphenicol-amended PDA at pH 5.5 or 7.0. Among the tested pH levels, pH 6.0 was the most favorable for *Mycosphaerella* isolation. PDA adjusted to pH 6.0 and supplemented with amoxicillin resulted in a 50% isolation success rate, with minimal contamination and pure *Mycosphaerella* isolates successfully obtained from three replicates without contamination by other fungi. Therefore, PDA at pH 6.0 supplemented with amoxicillin was selected as the optimal condition and subsequently used to evaluate the performance of other culture media (oatmeal agar and V8 juice agar) under two light regimes: a 12 h light/12 h dark photoperiod and 24 h continuous darkness. The results showed that oatmeal agar was a suitable alternative to PDA for supporting mycelial growth (Figure 3).

On oatmeal agar, *Mycosphaerella* exhibited greater mycelial growth than on the other two media. Modification of the light regime showed that oatmeal agar incubated under 24 h continuous darkness produced the highest mycelial growth rate (1.6 mm day^{-1}). Although this growth rate is relatively slow compared with those reported for other plant pathogenic fungi, such as *Fusarium* spp. and *Colletotrichum* spp., it is comparable to that of other *Cercospora* species, including *C. janseana*, the causal agent of narrow brown leaf spot of rice (Sa'adah and Tondok, 2022). However, the virulence of plant pathogenic fungi is not determined solely by mycelial growth rate but also by their ability to produce infective spores during the early logarithmic growth phase (Faway *et al*., 2021).

In addition to mycelial growth rate, conidial production is another critical parameter for determining the optimal growth conditions of fungal species with specific cultural requirements. The timing and abundance of conidial production are particularly important because conidia serve as asexual spores involved in infection, dispersal, and genome preservation under fluctuating environmental conditions (Osherov and May, 2001). In most fungi, conidia are produced within 24 h after incubation on agar blocks. In contrast, *Mycosphaerella* produced only newly emerging hyphae during the first 24 h of incubation. Conidiophores were first observed at 3 days after incubation (DAI), whereas young ascospores and conidia appeared at 5 DAI. Septation of the developing conidia began at 7 DAI.

Table 1. Colony establishment of *Mycosphaerella* sp. on potato dextrose agar at different pH levels supplemented with different antibiotics

Antibiotic treatment	Colony establishment of *Mycosphaerella* spp. (%) at different pH levels			
	5.5	6.0	6.5	7.0
Chloramphenicol	3.0 ± 5.8	30.0 ± 8.2	20.0 ± 0.0	0.0 ± 0.0
Amoxicillin	20.0 ± 10.0	50.0 ± 5.8	27.0 ± 15.3	20.0 ± 10.0

Note: Data are presented as mean ± SE (n = 10).

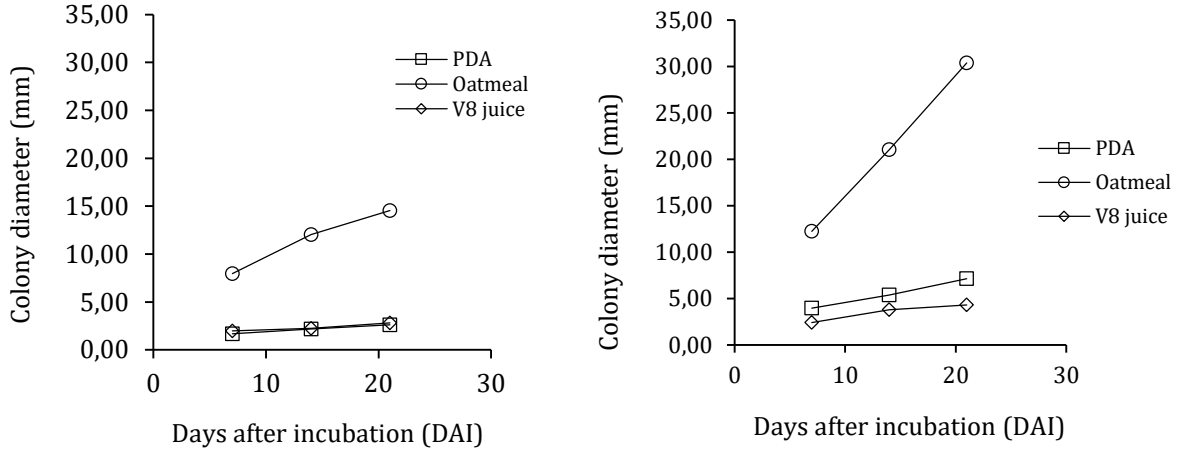

Figure 3. Colony diameter of *Mycosphaerella* sp. grown on potato dextrose agar (PDA), oatmeal agar (OA), and V8 juice agar under (a) a 12 h light/12 h dark photoperiod and (b) continuous darkness (24 h dark). Colony diameter was measured at 7, 14, and 21 days after incubation (DAI).

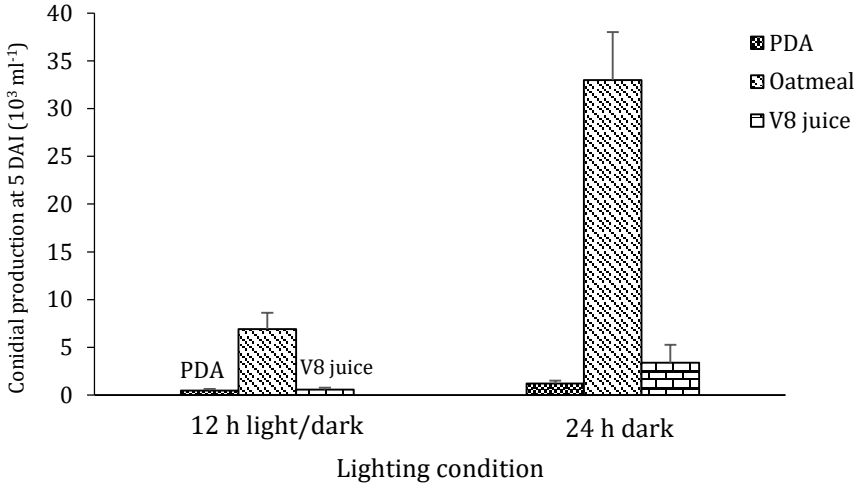

Figure 4. Effect of culture media and lighting conditions on ascospore and conidial production of *Mycosphaerella* sp. at 5 days after incubation.

In the *Mycosphaerella* species complex, the disease cycle comprises several stages, including inoculum production, spore dispersal, incubation, lesion development, and disease progression, all of which are influenced by environmental conditions (Poeydebat *et al*., 2018). Both ascospores and conidia play important roles throughout this disease cycle. Conidia primarily contribute to short-distance dissemination between leaves within the same plant or to neighboring plants through rain splash, whereas ascospores serve as the principal inoculum for long-distance dispersal by wind following rainfall events (Koppert, 2024). Zhang *et al*. (2005) reported that peak ascospore dispersal among different banana blocks and the initiation of new infection cycles may occur within 24 h after rainfall. For species identification, the production of conidia is particularly important because the morphological characteristics of asci and ascospores are relatively conserved among species within the phylum Ascomycota, making it difficult to distinguish *Mycosphaerella* from other genera based solely on sexual structures. The suitability of different culture media and incubation conditions for *Mycosphaerella* growth and conidial production is presented in Figure 4.

As shown in Figure 4, the highest conidial production was obtained on oatmeal agar supplemented with amoxicillin and incubated under 24 h continuous darkness for 5 days. Under these conditions, conidial production was approximately fourfold higher than that obtained on the same medium under a 12 h light/12 h dark photoperiod. In contrast, the opposite response has been reported for the oomycete *Hyaloperonospora arabidopsidis*. Telli *et al*. (2020) demonstrated that although continuous darkness promoted sporulation, the resulting spores exhibited poor germination. In contrast, optimal spore germination required a photoperiod of 10–14 h of light.

Rapid colony growth was not necessarily associated with greater conidial production, and vice versa. As shown in Figure 4, oatmeal agar consistently supported the highest conidial production under both lighting regimes. The greatest conidial yield was obtained on oatmeal agar incubated under 24 h continuous darkness. The stimulatory effect of darkness on colony development and sporulation observed in the present study may be associated with melanin biosynthesis, which is regulated by light intensity in many fungi (Beltran-Garcia *et al*., 2014). In contrast, continuous darkness did not enhance conidial production on PDA or V8 juice agar compared with the 12 h light/12 h dark photoperiod. These findings indicate that *Mycosphaerella* sp. exhibits a clear preference for oatmeal agar, with conidiation being further enhanced under continuous darkness. This preference is likely related to the nutritional composition of oatmeal agar, which is rich in carbohydrates, amino acids, peptides, vitamins, and minerals that support asexual sporulation in fungi, particularly members of the Ascomycota (Tan *et al*., 2019). In addition to commercially available synthetic oatmeal agar, researchers may also prepare the medium using 60 g L^{-1} oatmeal and 12.5 g L^{-1} agar, providing a practical alternative for routine laboratory culture.

In addition to nutrient availability, conidial production can also be influenced by biotic and abiotic stresses, or by interactions between the two (Leiva-Mora *et al*., 2008). For example, Nomiyama *et al*. (2024) reported that *Diaporthe destruens*, the causal agent of sweet potato stem rot, produced approximately 400-fold more pycnidia when grown on heat-treated sweet potato leaves than on agar medium without leaf tissue. These findings suggest that heat-stressed leaf tissues provide specific nutritional or physiological cues that promote fungal sporulation. These contrasting responses suggest that fungi possess adaptive mechanisms that enable them to respond to different environmental and nutritional cues. However, the genetic regulation underlying these adaptive responses remains poorly understood and warrants further investigation.

Collectively, the findings of the present study, together with previous reports, demonstrate that specific culture conditions are required to optimize mycelial growth and conidial production in *Mycosphaerella*. This finding is particularly important because *in vitro* conidial production in *Mycosphaerella* has frequently been reported to be inconsistent, representing a major limitation for studies requiring standardized inoculum. To overcome this constraint, Donzelli and Churchill (2007) developed a standardized mycelial fragment inoculation method for assessing the virulence of *M. fijiensis*. The optimized culture conditions identified in the present study may complement this approach by facilitating the production of conidial inoculum for studies in which reproductive propagules are required. Furthermore, the ability to reliably produce conidia under laboratory conditions is expected to facilitate future investigations into fungal biology, including studies on mutation, adaptation to biotic and abiotic stresses, and changes in virulence, which can be evaluated through conidial morphology, ascospore characteristics, and other phenotypic traits. Ultimately, these findings provide a useful methodological foundation for future research on *Mycosphaerella* biology and may contribute to the development of more effective strategies for the management of Sigatoka disease.

4. CONCLUSIONS

Leaves at the early stage of infection, characterized by brown streak lesions without a surrounding halo, were the most suitable inoculum source for isolating the *Mycosphaerella* species complex. Optimal fungal growth with minimal saprophytic contamination was obtained on oatmeal agar adjusted to pH 6.0 and supplemented with amoxicillin. Rapid mycelial growth and abundant conidial production were achieved when the fungus was incubated at room temperature under 24 h continuous darkness.

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