Effect of duration of mycelia fragmentation and concentration on virulence of *Mycosphaerella fijiensis* in banana

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Abstract

Mycelia from 3 isolates of *Mycosphaerella fijiensis* (Mak 01, Mak 02 and Kaw 10) were fragmented for 3 and 1 minutes, and tested at 5 mg mL^{-1}, 10 mg mL^{-1} and 15 mg mL^{-1} for virulence in the screen house. Disease severity was found to depend on the level of fragmentation and concentration. Inoculation of Gros Michel plantlets with mycelia fragmented for 3 minutes resulted in significantly higher levels of disease severity (AUDPC) than fragmentation for 1 minute for all the isolates. For both times of fragmentation, AUDPC increased with concentration. The highest and lowest AUDPCs for both times of fragmentation were achieved with concentrations of 15 and 5 mg mL^{-1}, respectively. Overall, all the three isolates demonstrated high levels of virulence, with Mak 01 causing the highest severity (AUDPC - 98.5) when compared to Mak 02 (AUDPC - 93.5) and Kaw 10 (AUDPC - 92.2) for 3-minute fragmentation and concentration of 15 mg mL^{-1}. Collectively, our data demonstrate that a potent inoculum of *Mycosphaerella fijiensis* for *in vitro* studies can be reliably prepared by fragmenting weighed mycelia in a blender.

Keywords: banana, *Mycosphaerella* genus, severity, area under disease progress curve.

Efeito da duração da fragmentação e concentração do micélio na virulência de *Mycosphaerella fijiensis* em bananeira

Resumo

Micélios de 3 isolados de *Mycosphaerella fijiensis* (Mak 01, Mak 02 e Kaw 10) foram fragmentados por 3 e 1 minutos, e testados em 5 mg mL^{-1}, 10 mg mL^{-1} e 15 mg mL^{-1} para virulência na casa de tela. Verificou-se que a gravidade da doença depende do nível de fragmentação e concentração. A inoculação de plântulas de Gros Michel com micélio fragmentado por 3 minutos resultou em níveis significativamente mais altos de severidade da doença (AUDPC) do que a fragmentação por 1 minuto para todos os isolados. Para ambos os tempos de fragmentação, AUDPC aumentou com a concentração. As maiores e menores AUDPCs para ambos os tempos de fragmentação foram obtidas com concentrações de 15 e 5 mg mL^{-1}, respectivamente. No geral, todos os três isolados demonstraram altos níveis de virulência, com Mak 01 causando a maior gravidade (AUDPC - 98,5) quando comparado com Mak 02 (AUDPC - 93,5) e Kaw 10 (AUDPC - 92,2) para fragmentação de 3 minutos e concentração de 15 mg mL^{-1}. Coletivamente, nossos dados demonstram que um inóculo potente de *Mycosphaerella fijiensis* para estudos *in vitro* pode ser preparado de forma confiável fragmentando micélios pesados em um liquidificador.

Palavras-chave: bananeira, gênero *Mycosphaerella*, severidade, área sob a curva de progresso da doença.

1. Introduction

Mycosphaerella fijiensis, the causal agent of black Sigatoka disease of banana is considered to be the most destructive leaf spot disease of banana (Churchill, 2010; Kumakech et al., 2015). It attacks all banana cultivars that belong to the AAA and AAB genome groups (Tushemereirwe et al., 2004; Churchill, 2011). The disease has

spread to various banana producing regions of the world (Carlier et al., 2000), including East Africa (Tushemereirwe et al., 2004) from the island of Fiji in South Pacific where it was first reported in 1963.

The assessment of virulence of *M. fijiensis* has several challenges (Donzelli; Churchill, 2007), including inconsistent conidia production and difficulties associated with causing controlled infections under ordinary screenhouse conditions (Balint-Kurti; Churchill, 2004; Noar; Daub, 2016). By increasing the reliability of inoculum production, *M. fijiensis* virulence assay efficiency could be improved. According to Donzelle & Churchil (2007), rapidity of symptom appearance is correlated with the amount of inoculum infecting banana plants. Twizeyimana et al. (2007) demonstrated the use of mycelia of *M. fijiensis* as inocula as opposed to conidia, but instead recommended the use of conidial suspensions because of the difficulty in quantification and standardization of mycelia suspensions.

Donzelli & Churchill, (2009) described a method of banana leaf inoculation that overcame the challenge of inconsistent conidia production by *M. fijiensis* using conidia. The method provided a way to determine virulence of strains of *M. fijiensis* using dose-response concept based on Area Under Disease Progress Curve data modelled using a General Linear Models (GLM) approach.

This approach however, required two parameters (the intercept and slope of each linear equation) to determine virulence of strains. Interpretation of data can be challenging, in the absence of a single numerical value with which to compare strains. Additionally, this method requires the use of expensive growth-chamber space for pathogenicity. This paper describes the effect of time of mycelial fragmentation and concentration on the virulence of *M. fijiensis* isolates under controlled conditions in Uganda using local humidity chamber innovation to facilitate infection.

2. Materials and Methods

2.1 Plant materials

Tissue culture banana plantlets of Gros Michel, a reference susceptible standard for *M. fijiensis* (Tushemereirwe et al., 2004) were used for screen house assays. Two-month-old seedlings obtained from Makerere University Agricultural Research Institute Kabanyolo (MUARIK) were transplanted to individual pots containing pre-sterilized loam soil. Banana plantlets were grown in the screen house at a temperature of 22-28 °C under natural light conditions for one month and used for experiments when they had 4-6 fully expanded leaves.

2.2 Mycosphaerella fijiensis isolates

Three *M. fijiensis* isolates (Mak 01, Mak 02 and Kaw 10) were isolated from banana fields in Wakiso district (Kawanda & Kabanyolo) using the procedure described by Stover (1976). Ascospore germination pattern was used to confirm the morphological feature of *M. fijiensis*. PCR - based molecular diagnostic assay was used to complement the morphological identification of *M. fijiensis*. Fungal cultures were maintained on potato dextrose agar (PDA, Himedia, India) at room temperature (25 °C).

2.3 Evaluation of mycelium concentration and duration of fragmentation

Inoculum for *M. fijiensis* isolates (Mak01, Mak02 and Kaw10) was prepared according to the procedure described by Donzelle and Churchil (2007) with slight modification. Agar disks, 3 mm in diameter, from actively growing PDA cultures of the isolates grown at 25 °C (< 15 days old), were aseptically transferred to malt extract agar (Oxoid, UK) plates using a sterile scalpel. 25 plates were prepared for each isolate. The inoculated plates were sealed and incubated at 25 °C for 14 days. Mycelium from each isolate were scraped off with a sterile scalpel, bulked and weighed in a pre-weighed sterile filter paper. The weighed mycelium was fragmented in a blender (commercial blender, USA) at full speed for 1 or 3 min. A master mycelium suspension was prepared for each isolate treatment and serial dilutions to final concentrations of 15, 10 and 5 mg mL^{-1} were made.

An inoculation experiment was conducted in the screenhouse at Muarik using a 3 x 2 x 3 factorial treatment structure in a Randomised Complete Block Design (RCBD) with three replications and repeated once. Factor one was *M. fijiensis* (3 isolates), factor two was mycelia fragmentation (3 and 1 min) and the third factor was mycelia concentrations (5, 10 and 15 mg mL^{-1}). The treatment consisted of inoculation of 3-month-old tissue cultured banana plants with fragmented mycelium of the three isolates at prescribed concentrations in steriled 0.18% water agar. Inoculum (1 mL) was applied to plants of Gros Michel (Bogoya) on the abaxial surfaces of the first and second fully unfolded leaves, until run-off, using a paint brush.

Control plants were inoculated with water. Each treatment was administered to three banana plantlets within one replicate. In both experiments, inoculated plantlets were incubated in a humidity chamber built using a timber frame covered with a translucent polythene sheet, at a temperature of 28-31 °C and continuous high relative humidity (approximately 90%), for 48 hours. High relative humidity was maintained by keeping 10 L of water in two open 5-litre plastic beakers. Temperature was monitored using a wall thermometer, placed inside the chamber. After the initial incubation, plants were transferred to a bench in the screen house and examined weekly for the first two weeks and subsequently daily for symptom development on both leaf surfaces for 21 days.

2.4 Data collection and analysis

Data on severity, incubation and latent periods were recorded. Severity was determined using the scale (0-5) described by Burt et al. (1999), where 0 = no spotting/necrosis, 1 = disease spots less than 5%, 2 = 5-15% necrotic tissue, 3 = 16-33% necrotic tissue, 4 = 34-50% necrotic tissue, and 5 = more than 50% necrotic tissue. Incubation period was measured as the period from inoculation to first symptom appearance and latent period as the period from first symptom appearance to full symptom development (lesion with grey centre surrounded by yellow hallow) (Marin et al., 2003). Severity data was used to calculate areas under disease progress curve (AUDPC) (Madden et al., 2007) prior to statistical analysis. All experimental data were subjected to analysis of variance (ANOVA) at significant level of 5%. All data analysis was performed using SAS (version 9.3, SAS Institute Inc., Cary, NC, USA).

3. Results

3.1 Symptom development

Black Sigatoka disease occurred for all three isolates on Gros Michel as presented in (Figure 1). Inoculated leaves developed necrotic tissue lesions. The first symptoms for all isolates were visible on average 24 days after inoculation (DAI). Symptoms manifested as light brown streaks that later became dark and coalesced. The disease lesions caused by the three isolates (mycelium suspensions of 15 mg mL^{-1}) were extensive at the end of the experiments (42 DAI), covering more than 80% of the inoculated leaves.

Figure 1. Necrotic lesions on Gros Michel plantlets caused by *Mycosphorella fijiensis* isolate; Mak 01 (**A**), Kaw10 (**B**) and Mak 02 (**C**). Source: Authors, 2023.

3.2 Virulence of isolates

Significant differences ($p < 0.05$) were observed in levels of disease caused by the three isolates when fragmented mycelia were used for inoculation. The effect of time of mycelial fragmentation and concentration were also significant ($p < .001$, $p < .001$ respectively). Similarly, the interaction terms: *M. fijiensis* x concentration, concentration × fragmentation time, and *M. fijiensis* x fragmentation time x concentration were significant ($p < .001$, $p < .001$, $p < .001$ respectively) (Table 1).

Inoculation of Gros Michel plantlets with mycelia fragmented for 3 minutes resulted in significantly ($p < 0.05$) higher levels of disease severity (AUDPC) when compared to fragmentation for 1 minute for all the isolates (Table 2). However, for both times of fragmentation, AUDPC increased with concentration. The highest and lowest AUDPCs for both times of fragmentation were achieved with concentrations of 15 and 5 mg mL^{-1},

respectively. The highest AUDPC values for 3-minute fragmentation were 98.5, 93.5, and 92.2 for Mak 01, Mak 02 and Kaw 10 respectively. Similarly, the highest AUDPC values for 1-minute fragmentation were 56.1, 54.4 and 48.2 for Mak 01, Mak 02 and Kaw 10 respectively. Overall, all the three isolates demonstrated high levels of virulence, with Mak 01 causing the highest severity (AUDPC - 98.5) when compared to Mak 02 (AUDPC - 93.5) and Kaw 10 (AUDPC - 92.2) for 3-minute fragmentation and concentration of 15 mg mL^{-1}.

Table 1. ANOVA for effect of mycelia concentration and time of fragmentaton on *M. fijiensis* virulence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication stratum	2	1.362	0.681	0.07	
M. fijiensis isolate	2	328.782	164.391	17.99	<.001
Fragmentation time	1	40716.75	40716.75	4455.35	<.001
Concentration	2	20548.512	10274.26	1124.24	<.001
M. fijiensis isolate x fragmentation	2	40.827	20.413	2.23	0.113
M. fijiensis isolate x concentration	4	263.139	65.785	7.2	<.001
Fragmentation time x concentration	2	352.905	176.452	19.31	<.001
M. fijiensis isolate x fragmentation x concentration	4	327.862	81.965	8.97	<.001
Residual	88	804.218	9.139		
Total	107	63384.357			

Source: Authors, 2023.

Table 2. Effect of isolate, mycelium concentration and time of fragmentation of *Mycosphaerella fijiensis* on AUDPC.

Isolate	Mycelia treatment combinations	Mean AUDPC values
Mak01	3 min fragmentation+ 15 mg mL^{-1}	98.5 ^{j}
	3 min fragmentation+ 10 mg mL^{-1}	72.5 ^{g}
	3 min fragmentation + 5 mg mL^{-1}	61.0 ^{e}
	1 min fragmentation + 15 mg mL^{-1}	56.1 ^{d}
	1 min fragmentation + 10 mg mL^{-1}	44.0 ^{b}
	1 min fragmentation + 5 mg mL^{-1}	20.3 ^{a}
Mak02	3 min fragmentation + 15 mg mL^{-1}	92.2 ^{i}
	3 min fragmentation + 10 mg mL^{-1}	77.6 ^{h}
	3 min fragmentation + 5 mg mL^{-1}	66.5 ^{f}
	1 min fragmentation + 15 mg mL^{-1}	54.4 ^{d}
	1 min fragmentation + 10 mg mL^{-1}	40.7 ^{b}
	1 min fragmentation + 5 mg mL^{-1}	20.4 ^{a}
Kaw10	3 min fragmentation + 15 mg mL^{-1}	93.5 ^{i}
	3 min fragmentation + 10 mg mL^{-1}	75.9 ^{g}
	3 min fragmentation + 5 mg mL^{-1}	54.0 ^{d}
	1 min fragmentation + 15 mg mL^{-1}	48.2 ^{c}
	1 min fragmentation + 10 mg mL^{-1}	40.5 ^{b}
	1 min fragmentation + 5 mg mL^{-1}	18.18 ^{a}
CV (%)		9.5

Note: ^{abc} Means followed by the same letter within the column are not significantly different ($p > 0.05$).

4. Discussion

Dependable and notable infection was achieved in the present study by applying weighed fragmented mycelium as inoculum on the entire leaf and incubating the inoculated plants in a locally fabricated humidity chamber for 48 hours prior to placing them on a screenhouse bench, without which no infection occurred in the screen house as was observed in the preliminary study. This method validated the reliability of *M. fijiensis* virulence assays under screen house conditions (room temperature, approximately 28 °C) in which weighed mycelia fragmented by a blender were used as inoculum for artificial inoculation, as opposed to mycelia fragmented by bead beating and quantified using a haemocytometer.

The current study provides a dose-dependent disease symptom development for the three isolates of *M. fijiensis*. Thus, the amount of inoculum of *M. fijiensis* for infection studies can be reliably measured in terms of weight. The weighed and fragmented mycelium-based approach for banana leaf inoculations of Gros Michel, a reference Black Sigatoka susceptible banana provided replicable and dependable results for the level of virulence of the three isolates *M.fijiensis* (Mak01, Mak02 and Kaw10).

Symptom development corresponded closely with those of *in vitro* plants (Grande Naine) inoculated with conidial suspension (Fullerton; Olsen, 1995). In spite of the variation in inoculum type and conditions from that of Fullerton & Olsen (1995), *M. fijiensis* disease development on the two susceptible cultivars (Gros Michel and Grande Naine) was comparable.

A potent inoculum of *M. fijiensis* for *in vitro* studies can, thus, be reliably prepared by fragmenting weighed mycelia in a blender. Black Sigatoka severity increased with increasing fragmentation and concentration because of increased number of infectious propagules (mycelia fragments). Quantification and standardisation of mycelia inoculum was possible, therefore, improving the experimental efficacy of *M. fijiensis*-banana pathosystem. It is, therefore, clear that weighed fragmented mycelia could be used to assess virulence of *M. fijiensis*, especially for isolates which sporulate poorly *in vitro*.

Variability in rate of disease development on inoculated leaves on the same plant treated with the same amount of inoculum was observed. Most likely, this was due to differences in age of the inoculated leaves. Although leaves of a susceptible banana are equally susceptible to *M. fijiensis*, most infections occur on new leaves. According to Churchill (2011), the same stages of black Sigatoka disease symptoms can vary, depending on leaf age at the time of infection.

The unanticipated difference in plant leaf response in these assays was taken care of by scoring the two inoculated leaves and using the average value for the two leaves in data analysis. It was also noted that incubation period in the emerging leaf was shorter than in fully expanded leaves. These findings suggest that younger leaves are more susceptible to fragmented mycelia than older leaves, and therefore, recommended for inoculation when using fragmented mycelia as inoculum. Susceptibility of younger leaves could have been due to the number of stomata, thereby facilitating entry of germinating mycelia.

The use of weighed fragmented mycelia as inocula has several advantages over that of conidia including reliability and reduced time needed to generate and prepare mycelia inoculum, and the ability to assess the virulence of isolates that sporulate poorly *in vitro*. Furthermore, the study significantly increased the reliability of *M. fijiensis* pathogenicity assays by shifting from the generally adopted strategy of use of expensive growth-chamber space for pathogenicity to local humidity chamber innovation to facilitate infection. In fact, increase in the chamber space would allow evaluation of more strains or isolates at the same time. This method therefore, will be of value for evaluating *M. fijiensis* pathogen virulence, as well as resistance in banana.

5. Conclusions

Black Sigatoka severity varied with time of mycelia fragmentation and concentration tested. Fragmentation of mycelia for 3 minutes and a concentration of 15 mg mL^{-1} generated the highest AUDPC values for the three isolates of *Mycosphaerella fijiensis*. Fragmentation of mycelia for 3 minutes and a concentration of 15 mg mL^{-1} is therefore, recommended for inoculum preparation.

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7. Auhors' Contributions

Alfred Kumakech: study design, experimentation, data collection, study writing, statistical analysis, publication. *Richard* Edema: experimentation, scientific reading, study writing, corrections and revisions. Patrick *Okori*: experimentation, scientific reading, study writing, corrections and revisions, and approval.

8. Conflicts of Interest

No conflicts of interest.

9. Ethics Approval

Not applicable.

10. References

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