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# Effect of Temperature on Germination, Radial Growth, and Sporulation of the New Isolates of *Metarhizium anisopliae* and Their Virulence to Whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae)

(Kesan Suhu terhadap Percambahan, Pertumbuhan Jejari dan Pensporaan Pencilan Baharu *Metarhizium anisopliae* dan Kevirulen terhadap Lalat Putih, *Bemisia tabaci* (Hemiptera: Aleyrodidae))

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ABSTRACT

*Metarhizium anisopliae* is a potential entomopathogenic fungi (EPF) to control insect pests in Malaysia, yet little is known about the temperature influence on its growth and efficacy. The average daily temperature in Malaysia is between 21 °C and 32 °C, with some parts of Cameron Highland in Peninsular Malaysia experiencing temperatures as low as 14 °C. Therefore, for effective field application, the most effective EPF isolates should be tolerant to the temperature of the country. Here we conducted a laboratory experiment to determine the effects of average daily temperatures in Malaysia (15, 20, 25, and 30 °C) on conidial germination, radial growth, and conidial density of seven new isolates of *M. anisopliae*. However, at 25 and 30°C, the three isolates (Ma-2, Ma-9, and Ma-15) produced the highest number of conidia and were therefore selected for virulence test against the second instar of whitefly, *Bemisia tabaci*. The percentage mortality of the three isolates ranged between 36.1% and 97.7% across different temperatures for 7 days post-treatment. The isolates Ma-15 caused the highest percentage mortality of 97.7% and the shortest $LT_{50}$ and $LT_{90}$ values (1.68 and 3.98 days, respectively), at 30 °C incubation temperature. This study confirms that the growth and virulence of Malaysian isolates of *M. anisopliae* are strongly influenced by temperature and could be used as promising candidates for biocontrol of *B. tabaci* on vegetables planted in a tropical climate such as that of low land and highland in Malaysia, although extensive field and semi-field bioassays, as well as the development of ideal formulation, are still required.

Keywords: *Bemisia tabaci*; entomopathogenic fungus; *Metarhizium anisopliae*; percentage mortality; temperature

ABSTRAK

*Metarhizium anisopliae* merupakan kulat entomopatogen (EPF) yang berpotensi untuk mengawal perosak serangga di Malaysia. Namun, pertumbuhan EPF bergantung pada suhu. Purata suhu harian di Malaysia adalah antara 21 °C dan 32 °C, dengan beberapa bahagian Tanah Tinggi Cameron di Semenanjung Malaysia mengalami suhu serendah 14 °C. Oleh itu, untuk aplikasi lapangan yang berkesan dan pencilan EPF yang paling berkesan ialah suhu setempat. Di sini, kami menjalankan uji kaji makmal untuk menentukan kesan purata suhu harian di Malaysia (15, 20, 25 dan 30 °C) terhadap percambahan konidium, pertumbuhan jejari dan ketumpatan konidium bagi tujuh pencilan baharu *M. anisopliae*. Walau

bagaimanapun, pada suhu 25 dan 30 °C, ketiga-tiga pencilan (*Ma*-2, *Ma*-9, dan *Ma*-15) menghasilkan bilangan konidium tertinggi dan oleh itu dipilih untuk ujian virulen terhadap instar kedua bagi lalat putih, *Bemisia tabaci*. Peratusan kematian ketiga-tiga pencilan adalah antara 36.1% dan 97.7% merentasi suhu berbeza selama 7 hari selepas rawatan. Pencilan *Ma*-15 menyebabkan peratusan kematian tertinggi iaitu 97.7% dan nilai $LT_{50}$ dan $LT_{90}$ terendah (masing-masing 1.68 dan 3.98 hari) pada suhu inkubasi 30 °C. Kajian ini mengesahkan bahawa pertumbuhan dan virulen pencilan *M. anisopliae* Malaysia sangat dipengaruhi oleh suhu dan boleh digunakan sebagai kawalan biologi yang berpotensi terhadap *B. tabaci* pada sayur-sayuran yang ditanam di iklim tropika seperti tanah rendah dan tanah tinggi di Malaysia, namun begitu, ujian lapangan dan semi-bioasai, serta pembangunan formulasi yang ideal masih diperlukan.

Kata kunci: *Bemisia tabaci*; kulat entomopatogen; *Metarhizium anisopliae*; peratusan kematian; suhu

## Introduction

*Metarhizium* (Hypocreales: Clavicipitaceae), also known as 'green muscardine fungus', is a genus of entomopathogenic fungi (EPF) that produce green conidia on the remains of their arthropod hosts (Nishi, Shimizu & Sato 2017). *Metarhizium anisopliae* is the most common species distributed worldwide and was the first known EPF to be used in controlling insect pests in the world (Brunner-Mendoza et al. 2019; Lord 2005). This species is widely used as an important biological control agent against various sap-sucking pests, including aphids, *Myzus persicae* (Sulzer), thrips, *Frankliniella occidentalis* (Pergande), and whitefly, *Bemisia tabaci* (Gennadius) (Antonio et al. 2012; Lee et al. 2015; Norhelina et al. 2013; Shan & Feng 2010) .

The whitefly *B. tabaci* (Gennadius) (Hemiptera: Aleyrodidae) has been recognized as one of the leading causes of damage to vegetables, greenhouse, and field crops around the world (Misaka et al. 2020). The insect is considered a cryptic species complex and an important polyphagous pest of more than 600 host plants (Shadmany et al. 2019). The two most invasive species of this insect; Biotype B and Biotype Q has been widely distributed in Malaysia and has been reported to cause significant yield loss to several vegetable crops such as tomato, chili, bell paper, and eggplant (Shadmany et al. 2019; Syed et al. 2000).

Biological control agents, such as *M. anisopliae*, have recently been identified as a potential alternative to synthetic chemicals in the control of *B. tabaci*. However, little research has been done on the impact of temperature on the efficacy of *M. anisopliae* against *B. tabaci.* Temperature tolerance of EPF, on the other hand, is shown to be relatively important because it affects fungal persistence and effectiveness, as well as shelf-life during storage (Tefera & Pringle 2003; Wu et al. 2020). It is therefore important to select fungi that are tolerant of the temperature for the development of more effective pest management in the agricultural ecosystem (Tefera & Pringle 2003).

To date, EPF research in Malaysia has tended to focus more on *M. anisopliae,* and application of it as a biological control for different insect pests has been widely demonstrated. Therefore, for effective field application, the most effective isolates should be tolerant to the range of temperature in Malaysia at which the targeted pest is more virulent. However, to establish a control strategy using fungi as a biological agent, it is necessary to explore the effects of Malaysian temperature on sporulation and growth for optimizing mass production of the EPF treatments.

The present study is therefore aimed to investigate the effect of temperature on growth and the virulence of newly isolated *M. anisopliae* isolates against *B. tabaci* under laboratory conditions.

## Materials and Methods

### Whitefly culture and host plant

The stock of whiteflies was collected in a glasshouse from tomato plants and reared at the glasshouse, Faculty of Agriculture, Universiti Putra Malaysia (UPM). The *B. tabaci* were morphologically identified by observing wings and bodies covered with a thin, powdery, or flour-like white wax (Hodges & Evans 2005), and using mitochondrial COI (mt COI) sequencing. The sequencing data were registered and assigned the accession number OM638559 (GenBank). The infested leaves used for bioassay were obtained by placing whitefly-free plants (21 days old) close to the adult-infested tomato seedlings for 72 h. Adult whiteflies were then removed, and seedlings infested with eggs were transferred to another glasshouse and placed in a cage for 12-15 days until the nymphs entered the second instar. This method resulted

in more than 40 eggs per leaf (Mascarin et al. 2013). *B. tabaci* population was maintained on tomato seedlings grown in 300 L pots and kept in cages at glasshouse condition (25-30 °C and 60-80% RH with a photoperiod of 12:12 h light: dark (L:D). Tomato plants were checked daily, and the damaged seedlings were replaced with new ones. The second nymphal instars were used for the virulence test because previous finding demonstrated that second instar of *B. tabaci* was highly susceptible to EPF infection (Mascarin et al. 2013; Sain et al. 2019).

### SOURCE OF FUNGAL ISOLATES AND CONIDIAL SUSPENSION

All the seven isolates of *M. anisopliae* used in this study were originally derived from soil samples collected from oil palm plantations of Universiti Putra Malaysia (Sani et al. 2022). The sequence data of the isolates have been deposited in the NCBI GenBank database and the details of the isolates can be seen in Table 1.

TABLE 1. Fungal isolates assessed in this study

| Isolates | Species | GenBank Accession | Origin | Hosts/sources |
|---|---|---|---|---|
| Ma-1B | *M. anisopliae* | MW857162 | UPM Serdang, Selangor Malaysia | Soil |
| Ma-2B | *M. anisopliae* | MW857163 | UPM Serdang, Selangor Malaysia | Soil |
| Ma-3E | *M. anisopliae* | MW857164 | UPM Serdang, Selangor Malaysia | Soil |
| Ma-4G | *M. anisopliae* | MW857165 | UPM Serdang, Selangor Malaysia | Soil |
| Ma-5i | *M. anisopliae* | MW857166 | UPM Serdang, Selangor Malaysia | Soil |
| Ma-9a | *M. anisopliae* | MW857167 | UPM Serdang, Selangor Malaysia | Soil |
| Ma-15 | *M. anisopliae* | MW857168 | UPM Serdang, Selangor Malaysia | Soil |

The conidia were harvested from a three-weeks-old culture grown on PDA slants by flooding 0.01% of tween 80 on sporulated cultures and scrapped using a sterile spatula (Ali et al. 2010). The conidia were suspended in a 50 mL plastic tube, vortexed for 2 min, and filtered through three layers of muslin cloth to remove debris and create a clean stock suspension.

### EFFECT OF TEMPERATURE ON CONIDIAL GERMINATION

The conidial germination for each isolate was assessed according to Onsongo et al. (2019), with slight modifications. The dimension of 7 $mm^2$ of each fresh isolate was cut with cork borer for recultured on PDA medium which would be further used for observation, including conidial density and viability of the seven isolates of the *M. anisopliae*.

The conidia from each isolate was counted under a light microscope (40×) using a Neubauer hemacytometer. For each of the isolates, 0.1 mL of $1\times10^6$ conidia/mL was evenly spread on Petri dishes (90 mm diameter) containing PDA slants. Three replicated sterile microscope coverslips were randomly placed on the surface of each plate. The plates were then sealed with parafilm and incubated in the darkness at 15, 20, 25, 30 °C for 24 h using three replications. The mean percentage germination of each fungal isolate was determined by randomly selecting 100 conidia and counting both germinated and non-germinated conidia beneath each coverslip under a light microscope (40×) (Onsongo et al. 2019).

### EFFECT OF TEMPERATURE ON RADIAL GROWTH

The radial growth of the seven isolates was determined by following the method of Ali et al. (2010). A plug of the mycelial tip with 7 $mm^2$ in diameter from the seven isolates was taken from 12 days old culture with a cork borer and aseptically plated on the central portion of the PDA media for the study of the growth rate and appearance of the mycelium. Plates were then incubated

at 15, 20, 25, 30 °C, and mycelial growth was measured at 3, 5, 7, 9, and 12 days by measuring colonies in two perpendicular directions (D1 and D2) on the bottom of each plate. Each of the isolates was replicated 3 times.

### EFFECT OF TEMPERATURE ON SPORULATION

All isolates' conidia were quantified on the 12 days based on their density in the inoculated culture. Conidia were obtained from 7 mm diameter mycelial discs and placed in a 15 mL tube having 10 mL sterile distilled water, which was vortexed for 3 min to obtain a homogeneous solution. Afterward, 1 mL of this suspension was picked and diluted through the additional 9 mL sterile distilled water and homogenized. Conidia were then counted under a light microscope (40×) using a Neubauer hemacytometer and expressed as conidial density (Sumikarsih, Herlinda & Pujiastuti 2019).

### EFFECT OF TEMPERATURE ON *M. anisopliae* VIRULENCE ON THE NYMPHS *B. tabaci*

The virulence test bioassay of *M. anisopliae* against whitefly nymphs was carried out to evaluate the efficacy of three isolates (Ma-2, Ma-9, and Ma-15) that produced the highest conidia and good performance on germination and radial growth on temperature tolerance were selected for virulence test against the second instar of *B. tabaci* under laboratory condition. Leaves infested with 20-30 nymphs were excised and dipped into a conidia concentration of $1\times10^7$. The excised leaves were air-dried and placed the abaxial surface up on the skinny layer of 1.5% agarose (Sain et al. 2019). The control excised leaves were dipped in 0.01% Tween 80, and all the treatments were incubated in a respective growth chamber at 15, 20, 25, and 30 °C. To prevent saprophytic fungi from growing on whitefly honeydew, aeration was established daily by opening each plate for 25- 30 min (Santiago-Alvarez et al. 2006). Dead nymphs (discoloured, and/ or desiccated, or become developed symptoms with fungal growth or sporulation) were recorded at 3, 5, and 7 days after incubation. The experiment was conducted 3 times with 3 replicates from each treatment.

### DATA ANALYSIS

The virulence of *M. anisopliae* against *B. tabaci* and the percentage of germination, radial growth, and conidial density were analyzed, using factorial analysis of variance (ANOVA) with R Software (version 3.6.1). The Abbott formula was used to correct the percent mortalities (Abbott 1925). Comparisons of the treatment means were performed using Fischer's least significant difference (LSD) test at $\alpha = 0.05$). The lethal time required to kill 50% and 90% ($LT_{50}$ and $LT_{90}$) were estimated for each replicate using the Generalized Linear Model (GLM).

## RESULT

### EFFECT OF TEMPERATURE ON CONIDIAL GERMINATION

The conidial germination of all the seven isolates was slowest and fastest at 15 °C and 30 °C incubation temperatures, respectively. The analysis of variance showed significant differences in percentage germination between fungal isolates and temperature treatments ($F= 17.65$, $DF=6$, $p=< 0.01$; $F= 9766.56$, $df= 3$, $p< 0.01$). However, there was significant interaction effect between temperature treatment and fungal isolates ($F = 7.69$, $df = 18$, $p < 0.05$). The highest percentage germinations of conidial were observed at temperatures of 25 and 30 °C (99%), which were not significantly different. However, the lowest mean conidia germination was observed at 15 °C regimes, which exhibit a significant percentage of germination at all incubation temperatures. A significant difference in germination by isolates was also observed at the 20 °C regimes which are ranged between 65.66% and 85.67% (Figure 1).

### EFFECT OF TEMPERATURE ON RADIAL GROWTH

An increase in fungal incubation temperature resulted in a significant increase in radial growth, with the lowest growth (12.00 mm±00) being recorded at 15 °C in Ma-4 isolate and the highest (71.00±0.69 mm) at 30 °C in Ma-2 (Figure 2).

A significant difference in mean radial growth was observed between fungal isolates at all temperature regime ($F = 7.86$, $df = 6$: $F = 608.32$, $df = 3$ $p< 0.01$). However, there was an interaction observed between fungal isolates and temperature ($F = 4.67$, $df = 18$, $p < 0.05$).

### EFFECT TEMPERATURE ON CONIDIAL DENSITY

The result of conidial production indicates that all isolates produced a higher number of conidia at 25 °C than at other incubation temperatures. The conidial yield of the Ma-9 isolate was much higher in comparison with other isolates. At all temperatures, Ma-3 sporulation was the slowest of the isolates examined (Figure 3).

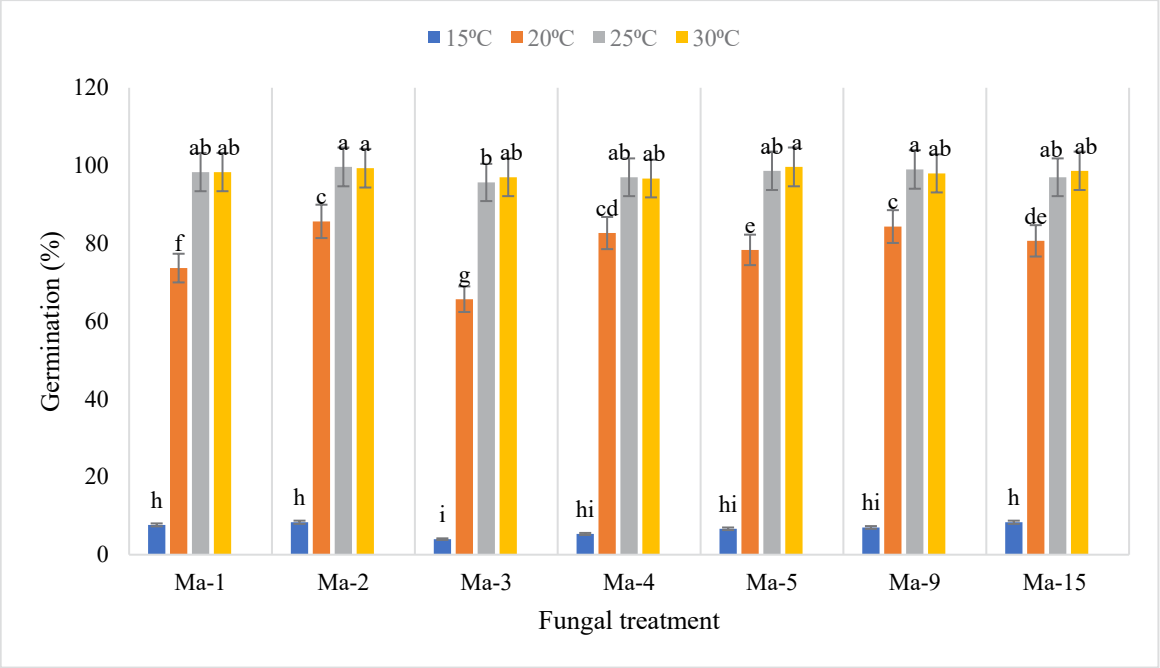


FIGURE 1. Effect of temperature on Conidia germination of *Metarhizium anisopliae* isolates. Bars with the same letter are not significantly different (α = 0.05) based on Fischer's least significant difference (LSD)

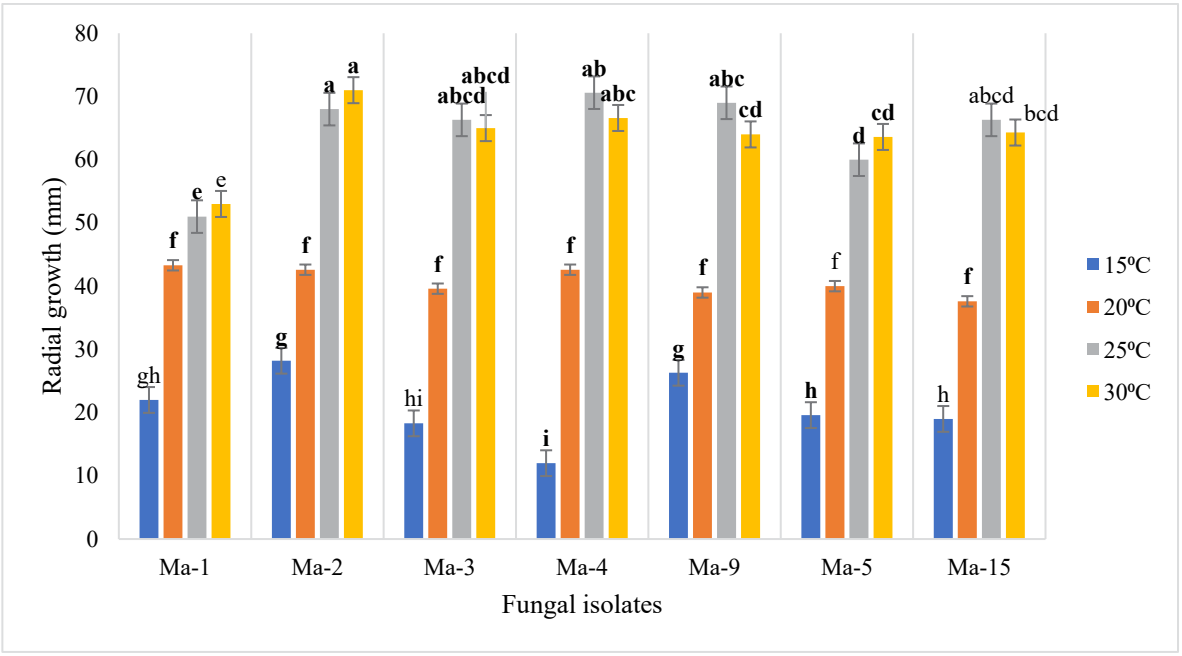


FIGURE 2. Effect of temperature on radial growth of *Metarhizium anisopliae* isolates. Bars with the same letter are not significantly different (α = 0.05) based on Fischer's least significant difference (LSD)

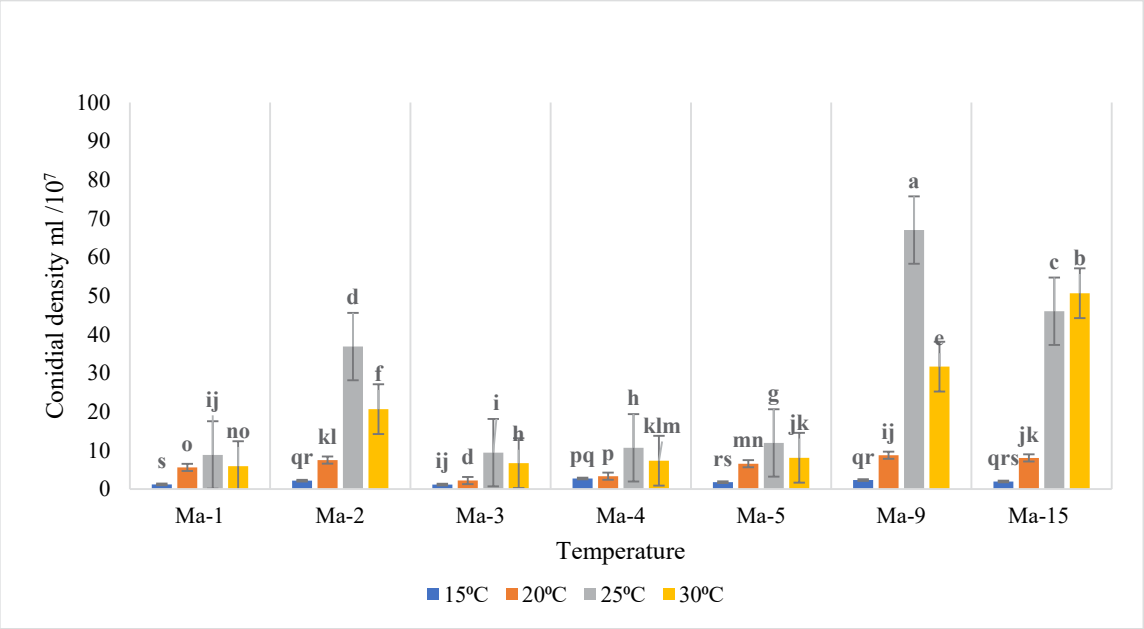


FIGURE 3. Effect of temperature on sporulation of *Metarhizium anisopliae* isolates. Error bars represent the standard error of the mean conidial density. Bars with the same letter are not significantly different (α = 0.05) based on Fischer's least significant difference (LSD)

The conidial density, like the other two growth parameters, significantly differed among isolates and incubation temperatures (F = 3897.22, df =6, $p < 0.01$; F=608.32, df =3, $p < 0.01$). There was also significant interaction effect between temperature treatment and fungal isolates in the production of conidia by the fungal isolates (F = 1322.45, df =18, $p < 0.01$).

### EFFECT OF TEMPERATURE ON VIRULENCE OF *M. anisopliae* ISOLATES TO *B. tabaci*

The result obtained from the fungal virulence test on *B. tabaci* indicated that all the three isolates were pathogenic to the second instar of *B. tabaci*. However, a significant effect of temperature and fungal isolates in causing mortality were observed in analysis of variance (F = 97.98, df = 3, $p = < 0.01$: F = 14.05, df = 2, $p < 0.01$).

Moreover, there was a significant interaction between the fungal isolates and temperature in causing mortality (F = 3.06, df = 6, $p = 0.02$). The highest mortality observed in all the three isolates, Ma-15 isolate (97.7%), Ma-2 (86.1%), and Ma-9 (84.6%) were recorded at a 30 °C incubation temperature (Figure 4). The lowest

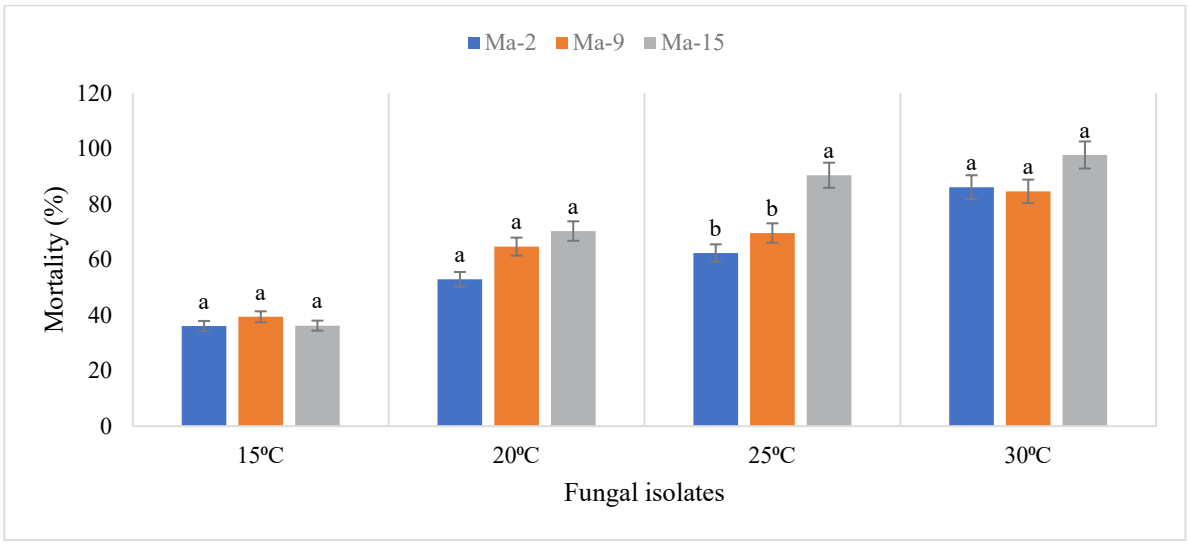


FIGURE 4. Mean mortality (%) of *Bemisia tabaci* at different temperatures for three isolates of *M. anisopliae* bars with the same letter are not significantly different (α = 0.05) based on Fischer's least significant difference (LSD)

mortality percentage of *B. tabaci* was observed at the 15 °C temperature regime with the isolates of Ma-2 having 36.1%, Ma-9a having 39.4, and Ma-15 having 36.2% (Figure 4).

For all the three isolates, the shortest $LT_{50}$ and $LT_{90}$ results were obtained at a 30 °C temperature regime (Table 2). Therefore, the Ma-15 isolate showed the highest efficacy in suppressing nymphs of *B. tabaci* at temperatures regimes of 25 and 30 °C which is optimal for fungal growth and sporulation.

TABLE 2. Lethal time to kill 50% and 90% of the second instar nymphs of *B. tabaci* caused by three *M. anisopliae* isolates at different temperature regimes

| | Ma-2b | | Ma-9a | | Ma-15 | |
|---|---|---|---|---|---|---|
| Temperature | $LT_{50}$ ± S.E (days) | $LT_{90}$ ± S.E (days) | $LT_{50}$ ± S.E (days) | $LT_{90}$ ± S.E (days) | $LT_{50}$ ± S.E (days) | $LT_{90}$ ± S.E (days) |
| 15 °C | 3.97 ±0.52 | 7.90 ±0.56 | 3.06 ±0.69 | 7.75 ±0.61 | 3.37 ±0.69 | 8.09 ±0.72 |
| 20 °C | 2.62 ±0.64 | 6.72 ±0.37 | 2.52 ±0.55 | 6.04 ±0.28 | 2.45 ±0.52 | 5.69 ±0.24 |
| 25 °C | 2.00 ±0.63 | 5.96 ±0.28 | 2.05 ±0.52 | 5.36 ±0.22 | 1.76 ±0.45 | 4.52 ±0.19 |
| 30 °C | 2.21 ±0.39 | 4.77 ±0.18 | 1.98 ±0.43 | 4.75 ±0.19 | 1.68 ±0.39 | 3.98 ±0.18 |

## DISCUSSION

The temperature influences all the entomopathogenic fungal parameters examined in this study, including virulence, conidial germination, radial growth, and sporulation. However, the optimal temperature for these growth parameters of *M. anisopliae* is generally found at 25 and 30 °C depending on fungal isolates. The isolate Ma-9 and Ma-2 resulted in the highest rate of conidia and radial growth at 30 °C incubation temperature, respectively. In contrast, the Ma-15 isolate caused the highest percentage mortality of 97.7% at 30 °C.

Similar finding have been demonstrated in different studies (Cabanillas & Jones 2009; Davidson et al. 2003; Sumikarsih, Herlinda & Pujiastuti 2019; Thomas & Jenkins 1997). For example, a significant positive correlation between these fungal parameters and virulence of *M. anisopliae* against *Zeugodacus cucurbitae* and *Zonocerus variegatus* at different temperatures have been reported (Onsongo et al. 2019; Susan et al. 2004; Thomas & Jenkins 1997).

Conidial germination of all isolates observed from different temperature regimes was influenced by temperature with over 90% germination recorded at 25 and 30 °C. Tefera and Pringle (2003), performed a similar experiment to show that *M. anisopliae* isolates produce more than 90% conidia at temperatures between 25 and 30 °C. Similarly, conidial germination of fungi isolates such as *B. bassiana* and *Isaria* sp. was found to be optimal at a similar temperature range (Cabanillas & Jones 2009; Sumikarsih, Herlinda & Pujiastuti 2019).

The radial growth of all fungal isolates was found at all temperature regimes but was significantly different between isolates, and the optimum temperature of growth recorded for all the isolates was 25 and 30 ºC. A study similar to this one that employed *M. anisopliae* was the findings of Onsongo et al. (2019) and Tefera and Pringle (2003).

Sporulation by EPF is an important parameter to consider when selecting fungal biocontrol agents of insect pests (Cabanillas & Jones 2009). Both temperature and fungal isolates have been found to influence spore density with the highest spore production recorded at 25 °C.

This finding is in accordance with that of Onsongo et al. (2019) and Tefera and Pringle (2003), whose found 25 °C to be the optimal temperature for sporulation of *M. anisopliae*. In this study, all the three isolates of *M. anisopliae* tested had a different pathogenic effect against *B. tabaci* and varied according to temperature regimes with the 25 and 30 °C being the most optimal in terms of *B. tabaci* percentage mortality. The previous finding by Taylor and Khan (2010), has shown that *M. anisopliae*

isolates were more susceptible to *B. tabaci* infection at 25 and 30 °C, which is consistent with our current study. The study of Keerio et al. (2020), is another example of the effect of different temperature on fungal infection to *B. tabaci,* which reduce the significant population at 24 and 32 °C.

Other research shows similar results when testing the virulence of *M. anisopliae* isolates at different temperatures against other insect pests such as *Maruca vitrata* (Lepidoptera: Crambidae) (Tumuhaise et al. 2018); *Chilo partellus* (Lepidoptera: Pyralidae) (Tefera & Pringle 2003), with the optimum temperature being 25 and 30 °C. As the temperature increases, the infection of *M. anisopliae* in *B. tabaci* increases. At 25, and 30 °C all fungal isolates were more virulent than at 20 and 15 °C, which is consistent with the previous studies (Susan et al. 2004; Taylor & Khan 2010).

A significant interaction was found between the temperature and the fungal isolates in causing mortality of *B. tabaci* and high mortality was recorded at 25 and 30 °C which is also optimal temperature for fungal growth. There have been several studies that showed the fungal pathogens cause high mortality as well as lower $LT_{50}$ and $LT_{90}$ at the optimum temperature for fungal growth which is ranged between 25 and 30 °C (Bugeme et al. 2008; Onsongo et al. 2019; Rodríguez, Gerding & France 2009). All fungal isolates have the same mode of infection process involving germination of spores on the cuticle of insects, penetration of hyphae on its body, multiplication of fungus in the body cavity, and death of insects (Inglis et al. 2001; Sani et al. 2020). At each stage of this infection process, a variety of enzymatic activities were known to operate (Ortiz-Urquiza & Keyhani 2013; Sandhu et al. 2012), suggesting that the effect of temperature on these enzymes systems differ as well, resulting in different optimum temperature.

It has been found that the effect of temperature on *M. anisopliae* virulence against insect pests varies depending on the geographic origin (Cabanillas & Jones 2009). For example, *M. anisopliae* isolates from the tropic region of the eastern part of Africa were found to be more effective on *Z. cucurbitae* at 30 °C, and predicted to be most effective in the tropical climates of South America and Africa and least effective in the United States of America and Canada (Onsongo et al. 2019). Consequently, the seven *M. anisopliae* isolates examined in this study were found in peninsular Malaysia, where tropical temperatures prevail.

## Conclusion

This study was conducted to characterize seven new isolates of *M. anisopliae* in terms of their tolerance to temperatures based on germination, growth, sporulation, and three among them for virulence to *B. tabaci*. The incubation temperature at 25 and 30 °C was extremely suitable to control whitefly. Furthermore, more research is needed to determine the effect of temperature on the virulence of EPF against *B. tabaci*, particularly in the field and semi-field. In addition to temperature, the effect of other factors such as humidity and ultraviolet rays on the growth performance of these EPF should be studied before field application.

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