Thermal Tolerance and Media Preferences of Entomopathogenic Fungal Isolates Virulent to *Pachnoda interrupta*

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Abstract

Chafer (*Pachnoda interrupta*) (Olivier) (Coleoptera: Scarabaeidae) is a serious pest of sorghum with a wide host range attacking over 37 crops in Africa in general and in Ethiopia in particular; and its control is solely dependent on synthetic insecticides. The cultural characteristics of five isolates of entomopathogenic fungi pathogenic to *P. interrupta* selected for their biological control were compared using the measurable parameters of germination, radial growth and sporulation on four (MEA, PDA, SDA and SDY) artificial media at five (15, 20, 25, 30, 35) different temperature levels. All the isolates, except PPRC2, achieved between 60-100% germination on all of the media used and at temperature levels of 20, 25 and 30 °C. PPRC2 germinated generally below 40% on PDA, SDA and SDAY. All of the abovementioned temperature levels supported better vegetative growth of the isolates in all of the media. The highest (31.17 mm) radial growth was recorded from MP3POST on SDA at 20 °C. Moreover, high spore production was recorded at these temperature levels with all the isolates producing above 2.23 x 10^{10} spores/mL. The highest number (8.77 x 10^{10} spores/mL) of spores was produced by IC-279 at 25 °C on SDA medium. All of the selected isolates exhibited good germination, radial growth and sporulation at temperature levels of 20, 25 and 30 °C on all of the tested media. However, relatively better radial growth and spore yield were obtained on SDA and SDAY media than on MEA and PDA at 30 °C. The two *Metarhzium anisopliae* isolates (PPRC51 and PPRC2) that had shown good performance with reference to in vitro characteristics are recommended as potential candidates for development of mycoinsectides against *P. interrupta*.

Keywords: Artificial media, germination, *Pachnoda interrupta*, radial growth, sporulation, thermal tolerance.

Introduction

Chafer (*Pachnoda interrupta* Olivier (Coleoptera: Scarabaeidae) is a serious pest of sorghum with a wide host range attacking over 37 crops and non-crop plants that belong to more than 20 families in Africa in general (Grunshaw 1992) and in Ethiopia in particular (Hiwot 2000). *P. interrupta* destroys sorghum fields completely during the outbreaks (Tsedeke 1988). The adult beetle of *P. interrupta* is the damaging stage and feeds on the flowers and sucks all the contents of sorghum, maize and wheat grains at the milk stage (Grunshaw 1992; Jago 1993; Troure & Yehouienou 1995). Yield losses ranging from 70 to 100% were recorded even on insecticide-treated sorghum fields (Yeraswork 2000; Yitbarek & Hiwot 2000). During the huge outbreaks in Ethiopia between 1993 and 2000, a single beetle on sorghum head was considered as an economic threshold to take management action (Hiwot MOA, Personal communication).

The current management methods against *P. interrupta* mainly depend on direct application of sprays and baits of synthetic insecticides (Seneshaw 2001). However, use of synthetic pesticides is not a sustainable method of pest management because of the detrimental effects on non-target organisms and the environment. Repeated use of pesticides may also induce pesticide resistance in target pests that, in turn, stimulates more frequent sprays at even higher concentration leading to more aggravated situations (Watkins et al. 2012).

On the other hand, developing and using rational biopesticides in an integrated pest management system has a potential to avoid the detrimental effects of agro-pesticides. Among the biorational pesticides, microbial biopesticides that include the ones developed from microorganisms, such as fungi, bacteria, viruses, protozoa and nematodes are the most commonly used bioagents (Gupta & Dikshit 2010). The microbial biocontrol agents (MBCAs) can destroy pests causing no detrimental side effects to human health and the environment and pest resistance development to MBCAs is less likely because of their complex multisite mode of actions (Khan et al. 2012). Entomopathogenic fungi are most preferred MBCAs because of a wide host range, ease of production and application, mode of action that does not need ingestion of the entomopathogen by the target pest and improvements in formulations (Butt 2002; Thomas & Read 2007; Wang & St. Leger 2007).

Developing microbial biopesticides in modern scientific studies demands characterization of selected microbial agents. The objective of this study was to determine the *in vitro* characteristics of selected entomopathogenic fungal isolates pathogenic to *P. interrupta* using measurable *in vitro* parameters on different artificial media and across five temperature regimes.

Materials and Methods

The experiments were conducted at the International Center of Insect Physiology and Ecology ICIPE) in Kenya. The cultural characteristics of five isolates of entomopathogenic fungi, i.e. three *Metarhzium anisopliae* (PPRC51, PPRC2, IC69) and two *Beauveria bassiana* (MP3POST, IC279) were compared using the measurable parameters of germination, radial growth and sporulation in four artificial media at five different

temperature regimes. Out of the five isolates characterized *in vitro*, IC69 and IC279 were obtained from ICIPE and were selected based on their performance on other insects (Ekesi et al. 1999; 2001) to serve as standards for comparison with the isolates from Ethiopia and were not tested for virulence against the target pest *P. interrupta*. The three isolates from Ethiopia (PPRC51, PPRC2 and MP3POST) were obtained from Ambo Plant Protection Research Center (APPRC). The isolates were previously tested against the target pest, *P. interrupta,* and caused 82.40, 77.14 and 79.63% mortality, respectively, and were selected from eight isolates on the basis of their virulence to *P. interrupta* (Belay et al. 2016). The treatments were arranged in a factorial experiment in a completely randomized design (CRD) with a 3 x 5 x 5 (media x isolate x temperature, respectively) treatment combinations.

Radial growth

Malt extract agar (MEA), potato dextrose agar (PDA), Sabouraud dextrose agar (SDA) and Sabouraud dextrose agar yeast (SDAY) were used as media for growth of the isolates. Conidial suspensions of the respective isolates were prepared by scrapping conidia with a sterile metal spatula from the surfaces of three-week old SDA cultures of the respective isolates and suspending them in 10 mL sterile water containing 0.05% Triton – X – 100. The concentration of the conidial suspensions was adjusted to 1 x 10^8 conidia/mL and 5 µl of the respective suspensions was immediately transferred after preparation to the center of each 8.5 cm Petri dish containing the respective media. The back of each Petri dish was marked with two perpendicular diameters that served as reference points for radial growth measurements. Each Petri dish served as a treatment and there were three replications per treatment. The inoculated media were then incubated at 15, 20, 25, 30 and 35 °C. The radial growth of each fungus isolate was measured every three days until 12 days after inoculation by taking the average growth in millimeters along two pre-drawn perpendicular reference lines on the back of the Petri dishes.

Germination assay

Media were prepared as in the radial growth assessment and 100 µL of a 1 x 10^6 conidia/mL suspension of the respective isolates was transferred to each medium and uniformly spread with a sterile glass rod throughout the Petri dish. These were then incubated at different temperature regimes as in the radial growth assessment. Germination was stopped after 24 hrs by applying 1 mL of lactophenol blue and three sterile cover slips were put in each medium. Percentage of germination was computed by counting at least 200 germinated and ungerminated spores under 400x magnification of a compound microscope.

Spore production

Media and conidial suspensions of the respective isolates were prepared as in radial growth and germination assay, respectively. Suspension of the respective isolates containing 1 x 10^6 conidia/mL was transferred to each medium at 100 µL per Petri dish and uniformly spread with a sterile glass rod throughout the Petri dish. These were then incubated at the different temperature regimes as in the radial growth evaluation. Three weeks after incubation, spores were harvested from the fully-sporulated cultures by scrapping conidia from the surfaces of three-week old cultures of the respective isolates and

media first with a sterile metal spatula and collecting in a sterile flask. This was then followed by washing the surfaces with two rounds of 50 mL of 0.05% Triton – X – 100 to fully dislodge the conidia to the flask. After vortex-shaking the suspensions for two minutes, the harvested conidia were counted using an improved neubaur haemocytometer

Statistical data analysis

Completely randomized design (CRD) with a 3 x 5 x 5 (media x isolate x temperature, respectively) factorial arrangement was used and three-way analysis of variance (ANOVA) was conducted on all of the data collected. Percentage data were arcsine transformed to normalize the variance before they were subjected to the ANOVA procedure of SAS statistical package version 9. Means were separated according to Tukey's Honestly Significant Difference (HSD) test.

Results

Germination assay

There was a highly significant difference in germination of the spores among all the isolates ($P < 0.0001$, df = 4, 200, F = 69.72) across all the temperatures ($P < 0.0001$, df = 4, 200, F = 1438.69) and all the media ($P < 0.0001$, df = 3, 200, F = 31.92). The interactions between isolates and media ($P<0.0001$, df = 12, 200, F = 30.59) isolates and temperatures ($P<0.0001$, df = 16, 200, F = 58.44) and among all the three factors ($P<0.0001$, df = 60, 200, F = 17.21) were also significant.

As shown (Figure 1), none of the isolates achieved 100% germination on any of the media at temperature levels of 15 and 35 °C except for MP3POST, which did so at 35 °C on MEA. All the isolates achieved between 60 and 100% germination on all the media and at temperature regimes of 20, 25 and 30 °C, except PPRC2, which germinated generally below 40% on PDA, SDA and SDAY. At 15 °C, all the isolates on all media showed below 27% germination except for PPRC51, which achieved 94% germination on SDA media. Relatively better germination was recorded at the highest (35 °C) temperature than at the lowest temperature (15 °C). At 35 °C, generally greater than 50% germination was achieved by all of the isolates on all of the media except for IC-69 (38% on MEA), IC-279 (21% on SDAY and 34% on MEA) and MP3POST (39% on PDA, 38% on SDA and 20% on SDAY).

Radial growth

The cumulative radial growths of the tested isolates of *B. bassiana* and *M. anisopliae* varied significantly among all the isolates ($P<0.0001$, df = 4, 200, F = 132.92) across all the temperatures ($P<0.0001$, df= 4, 200, F=817.9) and all the media ($P<0.0001$, df = 3, 200, F = 115.3). The interactions between isolates and media ($P<0.0001$, df = 12, 200, F = 21.74) isolates and temperatures ($P<0.0001$, df = 16, 200, F = 48.62) and among all the three factors ($P<0.0001$, df = 60, 200, F = 18.2) were also significant.

Results and Discussion

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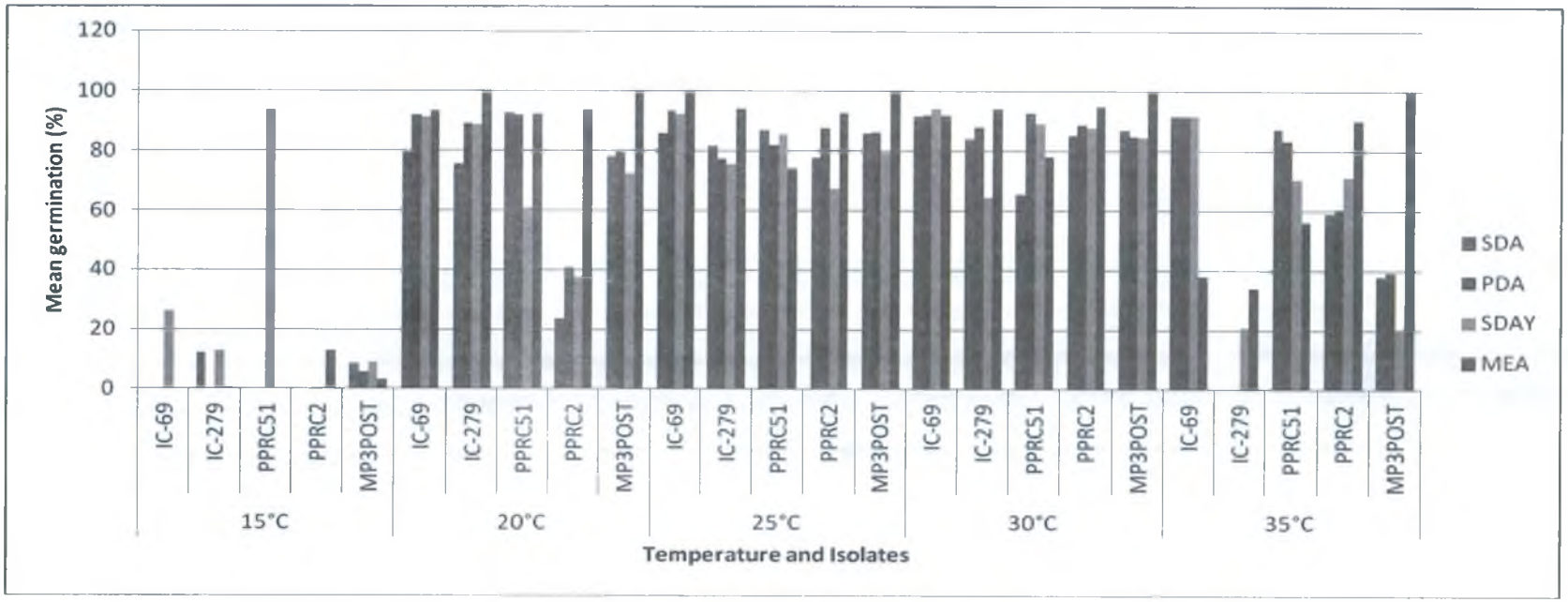

Figure 1. Germination of 2 *B. bassiana* and 3 *M. anisopliae* isolates on different artificial media and temperatures.

The mean values of radial growths of the isolates at the different temperature regimes and media are depicted hereunder (Figure 2). The radial growth of PPRC51 on SDA and PDA media at the respective temperatures is shown (Figure 3). The highest (31.17 mm) and lowest (0.33 mm) radial growths were recorded from MP3POST on SDA at 20 °C and SDAY at 35 °C, respectively. All of the isolates generally showed less than 10 mm of radial growth at the lowest (15 °C) and the highest (35 °C) temperatures in all of the artificial media except IC-69 (14.17 mm on SDA) and MP3POST (22.5 mm on SDAY). In contrast, temperature ranges of 20 – 30 °C supported better vegetative growth of the isolates in all of the media. The second highest radial growth was recorded from PPRC51 (30.83 mm) at 30 °C on SDAY media. This isolate also had better vegetative growth at this temperature on all the other media. Similarly, PPRC 2 showed the highest vegetative growth (19.83 mm) on the same medium (SDAY) at 25 °C.

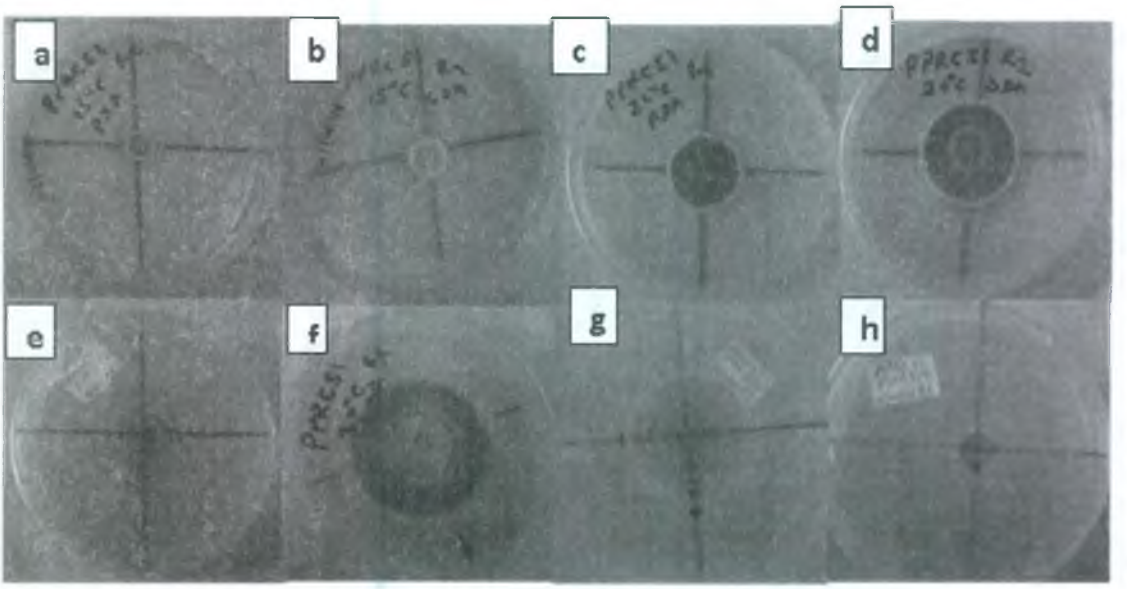

Figure 2: Radial growth and sporulation of PPRC51 isolate of *M. anisopliae* on SDA and PDA media at 15 °C (a and b), 20 °C (c and d), 25 °C (e), 30 °C (f and g), 35 °C (h) in 12 days.

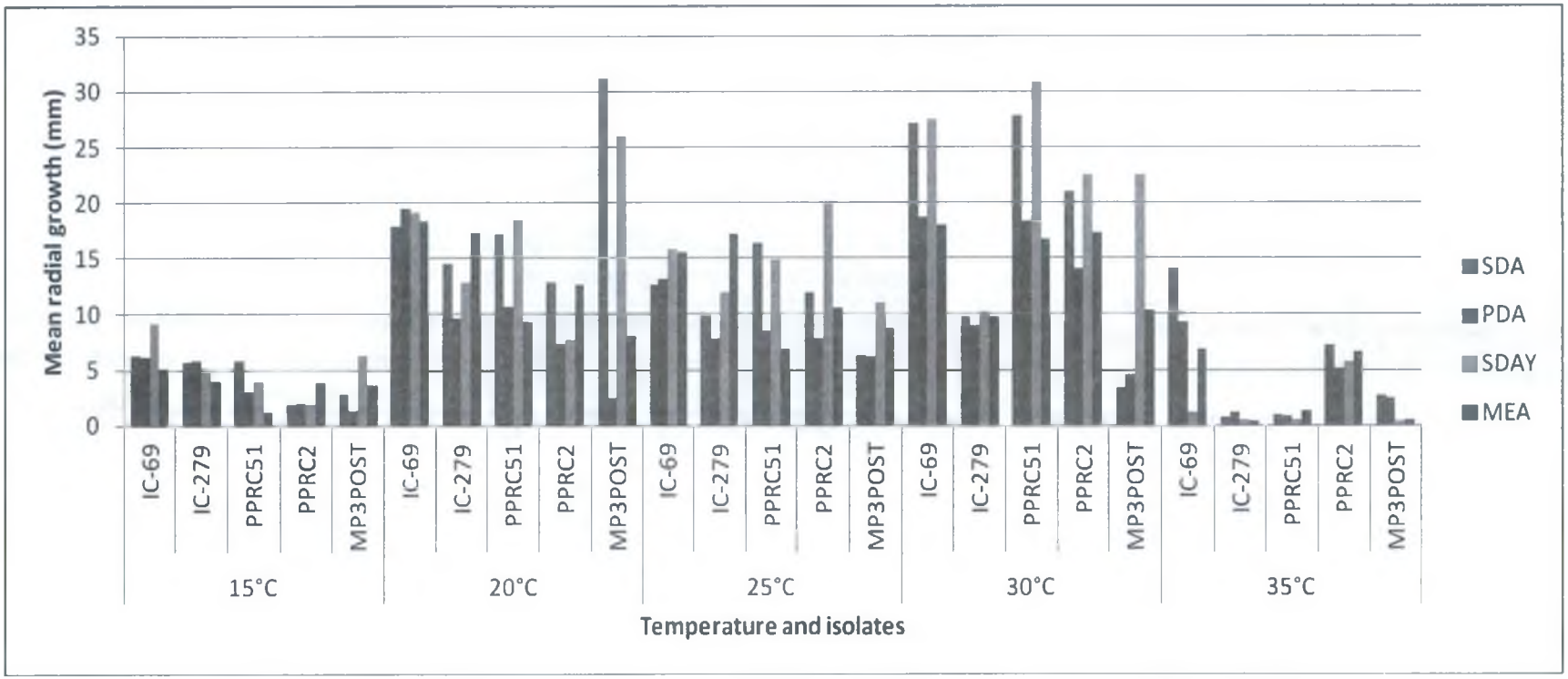

Figure 3. Radial growth of 2 *B. bassiana* and 3 *M. anisopliae* isolates on different artificial media and temperature levels.

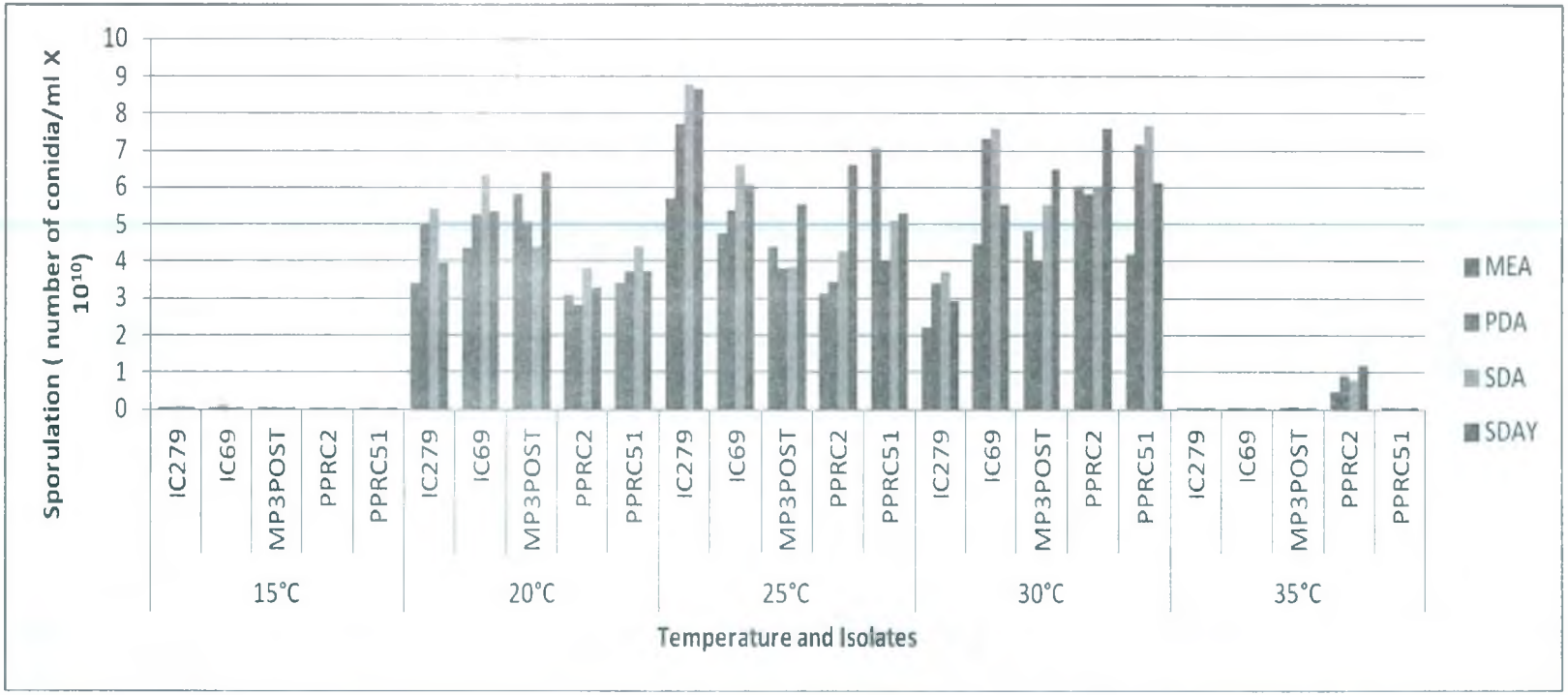

Figure 4. Sporulation of 2 *B. bassiana* and 3 *M. anisopliae* isolates on different artificial media and temperatures.

Evaluation of spore production:

The sporulation capacity of the isolates was evaluated based on the number of spores/mL. As in the radial growth and germination assay, highly significant variations were recorded in spore production among all the isolates ($P<0.0001$, df = 4, 200, F = 7.62) across all the temperatures ($P<0.0001$, df = 4, 200, F = 1525.25) and all the media ($P<0.0001$, df = 3, 200, F = 27.34). Significant variations were also recorded in the interactions between isolates and media ($P<0.0001$, df = 12, 200, F = 8.32), isolates and temperatures ($P<0.0001$, df = 16, 200, F = 45.93) and among all the three factors ($P<0.0001$, df = 60, 200, F = 5.26). The number of spores/mL produced at 15 and 35 °C were below 0.13×10^{10} and 1.2×10^{10}, respectively. High spore production was observed at temperatures of 20, 25 and 30 °C with all the isolates producing above 2.23×10^{10} spores/mL (Figure 4).

The highest number of spores was produced by IC-279 at 25 °C on SDA medium (8.77×10^{10} spores/mL) followed by the same isolate and temperature on SDAY (8.65×10^{10} spores/mL). The lowest number of spores (3.13×10^{10} res/mL) at this temperature was obtained from PPRC2 on MEA. At 30°C, the same isolate produced the lowest number of spore (2.23×10^{10} spores/mL) on MEA medium. On SDA media, PPRC51 produced the highest spores/mL (7.67×10^{10} spores/mL) followed by IC-69 (7.58×10^{10} spores/mL), while PPRC2 produced 7.6×10^{10} spores/mL at the same temperature on SDAY media. Two of the isolates from Ethiopia (PPRC51 and PPRC2) produced higher number of spores at 25 and 30 °C than at other temperature regimes and isolates. However, MP3POST produced comparable numbers of spores at 20 °C.

Discussion

Germination, sporulation and radial growth have been used in understanding the pathogenicity (Liu *et al.*, 2003) and characterization of entomopathogenic fungi (Almeida et al. 2005). Direct relationships between speed of infection of the host and increased radial growth have been suggested (Feijo et al. 2007). In addition to these, high spore production by the entomopathogen is an important factor for dispersal and epizootics (Mitchell 2003).

In this study the tests for germination, radial growth and sporulation at the different temperatures and media showed variations that indicated the relative thermal and nutritional preferences of the isolates. In general, spore germination was fairly high between 20 and 35 °C on all the artificial media. Similar findings were reported by other investigators (Tefera and Pringle 2003; Dimbi et al. 2004, Bugeme et al. 2008). However, germination of *B. bassiana* isolate IC279 was relatively lower at 35 °C, which is in agreement with the findings of Bugeme et al. (2008) who reported that *B. bassiana* isolates, including IC279, exhibited lower germination at this temperature than *M. anisopliae isolates*. In contrast, Ekesi et al. (1999) reported no significant difference in germination between isolates of *B. bassiana* and *M. anisopliae* at 15, 20, 25 and 30 °C, while differences occurred only at 35 °C. In this current study, however, significant differences were observed at these temperature levels across all the media. This can be attributed to nutritional and strain variability. Conidial germination, spore production

and other *in vitro* characteristics of entomopathogenic fungi are known to be affected by strain variability and nutritional compositions of the media, such as carbon sources, carbon to nitrogen ratio and their respective concentration (Leland et al. 2005; Shah et al. 2005).

Conidial germination was adversely affected by and rapidly slowed down at temperature levels above 30 °C with the optimum being between 23 and 28 °C and most entomopathogenic fungi isolates reducing to half at 34 - 37 °C (Jaronski 2010). Instability of conidia at high temperature indicates low thermo-tolerance (Fernandes et al. 2007). This necessitates the selection of isolates tolerant to the temperature range of the target ecosystem (Ferron et al. 1991). The two *M. anisopliae* isolates (PPRC51 and PPRC2) have exhibited relatively high germination at 35 °C which is a necessary characteristic for development of biopesticide against *P. interrupta* which inhabits and breeds in similar temperatures if the isolates can kill the pest at that temperature.

However, better radial growth was observed at temperature regimes of 20, 25 and 30 °C, while growth was limited at and below 15 and at and above 35 °C. Similar observation was also reported by Ekesi et al. (1999) who found low growth of *B. bassiana* and *M. anisopliae* at 15 and 35 °C without significant differences among isolates. Other researchers (Millner et al. 2002; Tefera and Pringle 2003; Rodriguez et al. 2009) have also reported limited radial growth of *B. bassiana* and *M. anisopliae* isolates at 35 °C on different media. Radial growth was generally below 19 mm in 12 days (1.58 mm day^{-1}) at 25 °C in all isolates despite use of different media in the study. Several studies have confirmed that the best radial growth of *M. anisopliae* and *B. bassiana* is generally achieved at temperature regimes between 20 and 30 °C (Ekesi et al. 1999; Tefera & Pringle 2003; Dimbi et al. 2004), which is in agreement with the result of the present study as well. In general, *M. anisopliae* isolates IC69 and PPRC51 showed better radial growth across all media at 30 °C.

Spore production was also restricted at 15 and 35 °C across all media. Relatively higher sporulation was observed at 30 °C than at 20 and 25 °C although there was variation among the isolates across the different media. Similar variation was observed among isolates of *M. anisopliae* (Niassy et al. 2012). In the present study, *M. anisopliae* isolates PPRC51, PPRC2 and IC69 gave the highest spore yield at 30 °C exhibiting better tolerance to high temperature levels.

The *in vitro* characterization indicated the best media and temperature for germination, radial growth and sporulation of the selected isolates. Thus, it is concluded that all the selected isolates exhibited best germination, radial growth and sporulation between temperature regimes of 20 and 30 °C on all the tested media. Maximum germination can be achieved using MEA media at 25 °C but, for cultivation of the isolates, use of SDA and SDAY media at 30 °C is most preferable as it supports better radial growth followed by substantial spore yield.

The two *M. anisopliae* isolates (PPRC51 and PPRC2) that showed good performance with *in vitro* characteristics are recommended as candidates for development of mycoinsectide against *P. interrupta.* However, further investigations are suggested to identify factors that enhance thermal tolerance and

infectivity of the isolates under field conditions.

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