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Effects of Water Activity and Temperature on Entomopathogenic Fungal Culture, Implications on Conidia Integrity and Virulence

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

Article Information

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ABSTRACT

Effects of available water for microbial growth, water activity (a_w) and incubation temperature on qualities of conidia produced by three entomopathogenic fungi, *Beauveria bassiana, Isaria farinosa* and *Metarhizium anisopliae* were evaluated *in vitro*. Sabouraud Dextrose Agar (SDA) (Sigma-Aldrich, 0.995 a_w) and SDA containing calculated amounts of glycerol were prepared to create different osmotically-stressed media (a_w = 0.98 or 0.96) and poured into 9 cm Petridishes. Agar plugs of seven days old cultures were transferred into the plates, sealed with parafilm and incubated at three different temperatures, 25, 30 and 35°C for 14 days. Harvested conidia from cultures grown at the three levels of interacting a_w and temperatures were described in relation to the growth conditions; A1-, A2- and A3-conidia, interacting growth conditions being 0.995 a_w x 25°C, 0.995 a_w x 30°C and 0.995 a_w x 35°C respectively. Similarly, B1-, B2- and B3-conidia (0.98 a_w x 25-35°C) and C1-, C2- and C3-conidia (0.96 a_w x 25-35°C). The conidia were tested for germination on different osmotically stressed media and virulence was evaluated using *Galleria*-model mortality bioassay. Germination rates of conidia was based on a 24-hour incubation period at 25°C while virulence was measured by the median lethal time (LT_{50}) against *Galleria mellonell*a larvae. There were significant variabilities in germination rates and virulence in relation to incubation temperature, osmotic status

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of media and the isolates. The A3-conidia of *Beauveria bassiana* and those grown on modified media, B2- and B3-conidia, failed to germinate after 24 hours of incubation, and they were less virulent. Similarly, B3- and C3-conidia of *M. anisopliae* could not germinate after 24 hours. In contrast, all *I. farinosa* propagules germinated, although the non osmotically-stressed A1-, A2- and A3-conidia had relatively higher germination rates. Generally, the non-osmotically stressed conidia performed better in terms of germination and virulence. This is a model study on the optimal culture conditions for production of conidia of the entomopathogens in Submerged Fermentation (SF) systems.

Keywords: Germination rate; virulence; entomopathogenic fungi; Galleria mellonella; conidia.

1. INTRODUCTION

There is significant interest in developing alternative pest control methods, which are capable of reducing dependence on chemical insecticides in integrated pest management (IPM) systems. Entomopathogenic fungal species, especially isolates of *Beauveria bassiana, Metarhizium anisopliae* and *Isaria farinosa* have emerged as potential biocontrol agents [1,2,3] and commercially available fungal based biopesticides have shown effectiveness against important insect pests [4].

A major consideration in the development of entomopathogenic fungi for biological control is the selection of the most virulent strains against the target pest [5]. Abadias et al. [6] suggested that, for a fungal biocontrol agent to be suitable for use under field conditions, it is important that the inoculum has necessary ecological fitness to withstand abiotic interactions in form of natural fluctuations in temperature and relative humidity. Borisade [1] reported that, practically under field conditions, success of entomopathogenic biocontrol agents depend on their ability to exploit short favourable periods for growth and infection of their hosts. When native species are used, biocontrol microorganisms are naturally fit to survive in their environment. However, studies on ecophysiology of nineteen strains of entomopathogenic *Beauveria bassiana, Isaria farinosa, I. fumosorosea* and *Metarhizium anisopliae* from different agro-ecological regions (tropical and temperate) have shown that most isolates have narrow relative humidity and temperature windows within which they can grow, sporulate [7] and be infective [1].

Thus, much research efforts are directed towards improvement of strains through manipulation of growth conditions to increase the viability of cells and improve resilience to interacting abiotic stress factors. Van Eck et al. [8] showed that most organisms accumulate low molecular mass compounds as a physiological mechanism to equilibrate their cytoplasmic water activity (a_w) with the a_w of the environment when they are exposed to osmotic stress. Similarly, Hallsworth and Magan [9] reported that *M.anisopliae* cultures grown on glycerol modified-Sabouraud Dextrose Agar (SDA) based media with low water activity (0.98 a_w) produced conidia with higher percentage germination under abiotic stress factors. The reports of Hallsworth and Magan [9] compared the effect of a_w modifying solutes on ecological fitness and integrity of conidia and concluded that glycerol, which is a utilisable carbon source, produced better results compared with ethylene glycol (EG600). Jackson and Jaronski [10] showed that Carbon to Nitrogen (C: N) ratio can be adjusted in submerged culture to improve germination rates of blastospores of *Metarhizium anisopliae*.

Several other studies have demonstrated that growth on different carbon sources which are capable of modifying a_w of the media enhance accumulation of glycerol and erythritol into mycelia and conidia, thereby improving qualities of propagules in terms of tolerance to abiotic stress factors [11,12,13]. However, the effects of two-way interactions between media a_w and incubation temperatures on entomopathogenic fungal culture and the implications on germination and virulence of conidia have not been widely reported. Conidia of entomopathogenic fungi harvested from cultures grown at different levels of osmotic stress and incubation temperatures need to be tested for infectivity and virulence. This kind of information is necessary for the improvement of culture conditions to produce conidia that combine tolerance to abiotic stress factors with high infectivity.

The larvae of the greater wax moth, *Galleria mellonella* has been employed in the study of microbial-host interactions and evaluation of pathogenicity or infectivity with reliable results. It

has gained popularity as an insect model for studies on the virulence of entomopathogenic fungi because of its relatively handy size and ease of culture under laboratory conditions. Positive correlations between virulence of microbial candidates to target hosts have been established using *Galleria* model studies [14,15, 16].

In this study, SDA was modified using calculated amounts of the non-ionic solute, glycerol to create different osmotic stress (a_w) conditions in media. Strains of *B. bassiana, I. farinosa* and *M. anisopliae* were cultured on these media and samples were incubated at different temperatures. Thereafter, harvested conidia were tested for germination during a 24-hour incubation period and virulence of the infective conidia was evaluated using *G. mellonella l*arvae model mortality assay under optimal equilibrium relative humidity (ERH) condition. The aim was to understand the impact of incubation temperatures and a_w of growth media on quality of conidia in terms of germination rates and virulence.

2. MATERIALS AND METHODS

2.1 Source of Fungi and *G. mellonella* larvae

B. bassiana (bb 315) and *M.anisopliae* (Ma V275) isolates were generously supplied by the International Institute of Tropical Agriculture (IITA), Republic of Benin, while *I. farinosa* (ARSEF 5081) was provided by the United States Department of Agriculture, Agricultural Research Service. Third instar larvae of *G. mellonella* were extracted from infested honeycombs at the Teaching and Research Farms, Crop Bio-protection Unit, Faculty of Agricultural Sciences, Ekiti State University, Nigeria.

2.2 Laboratory Maintenance of *G. mellonella* larvae

About three hundred larvae were kept in insect rearing cage at 20°C inside laboratory to delay pupation. A glass jar containing 500 ml deionised water was placed inside the insect cage for equilibration (>98% ERH) to reduce mortality. They were fed on antibiotic-free cereal-honey based diet compounded by mixing 176 g maize grit, 235 g rice bran, 10 g yeast granules, 65 g wheat grit, 70 g rice grit, 75 ml honey (Fresh local honey) and 90 ml glycerol until friable and packed into sealable polythene bags for storage at 4°C. Food was made available ad-libitum and the larvae were used for bioassay within 10 days of rearing.

2.3 Fungal Culture, Harvesting and Description of Conidia

One centimetre agar plugs from 14 days old culture of each isolate was subcultured in triplicates on unmodified standard SDA (Sigma-Aldrich, 0.995 a_w) or SDA media containing calculated amounts of glycerol to create different osmotic stress levels (a_w = 0.98 or 0.96). The inoculated plates were sealed with parafilm to prevent moisture evaporation from the agar surface and incubated at three different temperatures, 25, 30 and 35°C for 14 days. Isotonic solutions (0.995, 0.98 and 0.96 a_w) were prepared with sterile Reverse Osmosis (RO) water containing 0.02% Tween 80 and glycerol. Conidia were scrapped into 10 ml solution that is isotonic to the a_w of the growth media (Hallsworth and Magan, 2006) and the concentration was adjusted to 1 x 10^6 conidia ml^{-1} using Neubauer haemocytometer. The three levels of water availability, 0.995, 0.98 and 0.96 a_w were represented with letters A, B and C while the incubation temperatures, 25, 30 and 35°C were represented by numbers 1, 2 and 3 respectively. Thus, conidia were described in relation to the a_w of the media from which they were harvested and the incubation temperature of the culture as shown in Table 1.

2.4 Evaluation of Conidia Germination Rates

Germination rates were assessed by spread-plating conidia suspension containing 1.0 x 10^4 conidia ml^{-1} on standard SDA media in 9 cm Petridishes in triplicates. The Petridishes were sealed with parafilm and incubated for 24 hours at 25°C in the dark. Thereafter, sterile coverslips were placed at three positions on the media in each Petridish and random counting was done for 50 conidia within each cover slip area under microscope using x40 objective. A conidium with developed germ tube was considered as

Table 1. Description of fungal conidia harvested from cultures grown under three different a_w and incubation temperatures

Media water a_w levels	Alphabets representing a_w levels	Incubation temperature levels (ITL)	Numbers representing ITL	Description of conidia harvested from cultures (Media a_w x ITL)
0.995 a_w	A	25 °C	1	A1
0.995 a_w	A	30 °C	2	A2
0.995 a_w	A	35 °C	3	A3
0.98 a_w	B	25 °C	1	B1
0.98 a_w	B	30 °C	2	B2
0.98 a_w	B	35 °C	3	B3
0.96 a_w	C	25 °C	1	C1
0.96 a_w	C	30 °C	2	C2
0.96 a_w	C	35 °C	3	C3

germinated. The mean percentage germination was calculated as shown in equation one below:

Percentage germination =

$$\frac{Germinated\ conidia}{Total\ cou\ ntedconidia}\ X\ \frac{100}{1}$$

2.5 Evaluation of Conidia Virulence

Assessment of virulence of conidia using *Galleria* bioassay system was performed using 9 cm Petridish with 1 cm^2 ventilation hole on the lid, covered with muslin cloth. Whatman filter paper was placed in the dish and *G. mellonella* larvae was dipped into prepared conidia suspension containing 1 x 10^6 conidia ml^{-1} for 1-2 seconds before placing on the filter paper in the Petridish. The filter paper was placed to absorb excess water on the larvae in order to prevent suffocation due to spiracle blockage. A total of ten larvae per Petridish was used. The control consisted of ten larvae samples dipped in sterile de-ionised water containing 0.05% Tween-80. The set-up was replicated 3 times and the Petridishes containing the inoculated larvae and the control were arranged separately inside plastic boxes measuring 30 x 30 x 25 cm^3. The lid of each plastic box had 25 cm^2 ventilation hole lined with muslin cloth to allow aeration. Two beakers, each containing 500 ml sterile de-ionised water was placed inside the box for equilibration (equilibrium relative humidity, ERH>99%) at 25°C to enhance infection and cumulative mortality of the larvae was recorded every 24 hours for 5 days (Borisade, 2016). Single conidia concentration (1 x 10^6 conidia ml^{-1}) was used for the bio-assay and virulence of conidia was measured by the Median Lethal Time (LT_{50}).

3. STATISTICAL ANALYSIS

Galleria mellonella mortality data was not corrected for natural mortality because there was less than 10% mortality in control. Probit values of the percentage cumulative mortality against time was plotted and LT_{50} was determined from the regression equation of the curve. The data on conidia germination and LT_{50} were subjected to analysis of variance (ANOVA) procedure. Where significant differences were found, means were separated using Tukey's Honestly Significant Difference (P=0.05). Data analysis was done using the IBM-SPSS 21 statistical software. Bar graphs were plotted using MS Excel 2010and quantitative values of LT_{50} (days) and germination (%) were indicated.

4. RESULTS

4.1 Temperature and a_w Boundaries for Conidia Production

The conidia (A1, A2....C3) were harvested from cultures grown on PDA at three different levels of water availability and incubation temperatures as described in Table 1. It was found that lower a_w boundary and upper temperature limits at which the isolates of *B. bassiana* and *I. farinosa* could be cultured for production of conidia were 0.96 a_w at 25°C, C1 and 0.96 a_w at 30°C, C2 respectively. At higher temperature levels, there was no significant growth on inoculated plates. In contrast, *M. anisopliae* grew and produced conidia over a relatively wider a_w and temperature ranges, 0.995-0.96 a_w at 25-35°C.

4.2 Effect of Media a_w and Incubation Temperature on Germination and Virulence of Conidia

Incubation temperature and a_w of growth media differentially affected the rates of germination of harvested conidia after 24 hours incubation as well as virulence. The set of *B. bassiana* conidia, A1- and A2-conidia, harvested from cultures grown on media with freely available water (0.995 a_w) at 25-30°C had significantly higher percentage germination, being 95 and 94% respectively after 24 hours incubation (Fig. 1a). The mean percentage germination of B1-conidia (Culture growth condition: 0.98 a_w at 25°C) was 94% and this was not significantly different from the A1- and A2-conidia. Incubation of *B. bassiana* culture growing on media with freely available water at 35°C temperature produced dormant conidia that failed to germinate after incubating at 25°C for 24 hours. B2-and B3-conidia, culture growth conditions being 0.98 a_w at 30 and 35°C respectively also failed to germinate after a 24-hour incubation period at 25°C. The germination rate of C1-conidia (culture growth condition: 0.96 a_w and 25°C) was significantly the lowest. In terms of virulence, A1- and A2-conidia were not significantly different from each other, LT_{50} values being 2.9 and 3.2. Other conidia, B and C sets had significantly higher LT_{50} values Fig. 1b.

Germination rates and virulence of *I. farinosa* are shown in Figs. 2a and 2b respectively. There were significant variabilities in germination rates of the three sets of conidia; A, B and C. Apart from *B. bassiana*, none of the sets of conidia had zero percentage germination after 24 hours incubation. Significantly, higher germination rates were recorded in A1-, A2- and B1-conidia. The cultures grown at 0.98 a_w and 35°C as well as 0.96 a_w and 25-35°C produced conidia with relatively poor germination rates and the effect of 35°C incubation temperature on quality of conidia was dramatic and negative. The LT_{50} of A1-conidia (1.15 days) was significantly the lowest, followed by A3-conidia (2.1 days). Virulence of A2- and C1-conidia were not significantly different, LT_{50} values being 2.7 and 2.9 days respectively.

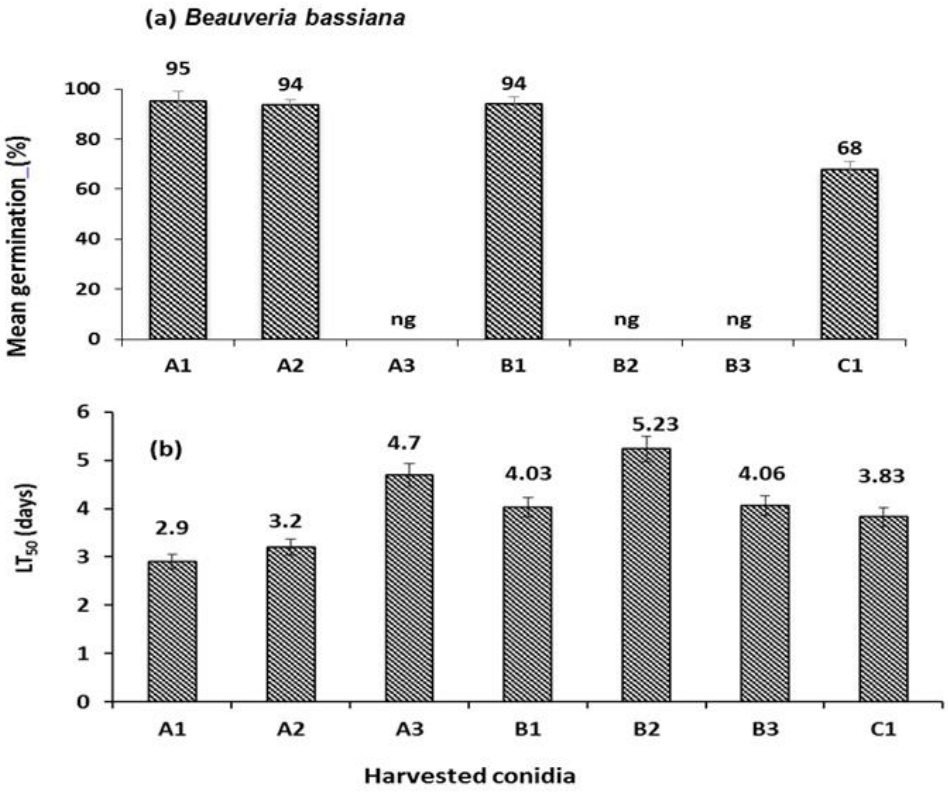

Fig. 1. Effect of media a_w and incubation temperature on quality of *B.bassiana* conidia in relation to (a) germination rate and (b) virulence.ng=no germination

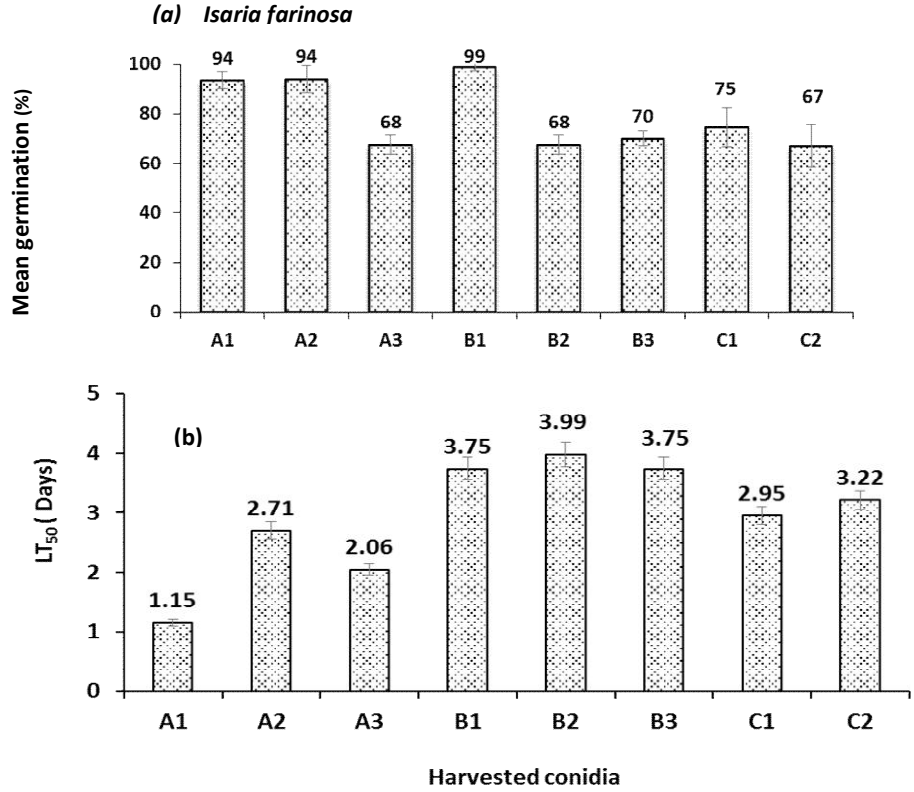

Fig. 2. Effect of media a_w and incubation temperature on quality of *I. farinosa* conidia in relation to (a) germination rate (%) and (b) virulence

Germination rates of A1-, A2-, A3-, B1- and B2-conidia of *M. anisopliae* were between 94-97% and they were not significantly different from each other. However, B3 and C3-conidia failed to germinate after 24 hours incubation period at 25°C (Fig. 3a). Regarding virulence, A1- and A3-conidia had significantly lower LT_{50} values, which were 4.8 and 5.1 days respectively (Figure 3b) and the isolate of *M. anisopliae* was less virulent compared to *B. bassiana* and *I. farinosa.* The optimal growth conditions for production of fast-germinating and virulent conidia differed for each isolate and were between 0.995-0.98 a_w and 25-30°C.

5. DISCUSSION

Conidia from cultures grown on glycerol-modified media are herein referred to as modified propagules or modified conidia while those from the medium with freely available water (non-osmotically stressed) are the unmodified propagules. This study has shown that manipulation of growth conditions by changing the a_w of media using non-ionic solute glycerol significantly influenced rate of germination and virulence of entomopathogenic *B. bassiana, I. farinosa* and *M.anisopliae* conidia. Single effect of incubation temperature on growth, where water was freely available in the media and its interaction in the presence of a_w modifying solute dramatically influenced conidia germination rate and virulence.

Beauveria bassiana conidia harvested from cultures grown at 35°C and 0.995 a_w (A3-conidia) and those grown on modified media, 0.98 a_w and incubated at 30-35°C; B2- and B3-conidia, failed to germinate after 24 hours of incubation and were less virulent. Similarly, B3-conidia and C3-conidia of *M. anisopliae* (harvested from cultures grown at 0.96 a_w and 35 °C) could not germinate after a 24-hour incubation period; they also had relatively longer LT_{50} compared with the non-osmotically stressed conidia. In contrast, *I. farinosa* conidia germinated differentially (64-97% germination) after incubation for 24 hours at 25°C. However, non-osmotically stressed A1-, A2- and A3-conidia had relatively higher germination rates and lower LT_{50}. Abadias et al. [6] grew a strain of biocontrol yeast, *Candida sake* at 0.98 and 0.96 a_w in molasses using

different a_w modifying solutes at 25°C and reported that significant differences existed in intracellular accumulation of solutes but conidia viability was not significantly affected. Rangel et al. [17] similarly evaluated the effect of physical and nutritional stress conditions during mycelia growth on conidia germination rate, adhesion to host cuticles and virulence of *M. anisopliae,* and showed that osmotically stressed and nutrient-poor media produced more virulent conidia.

There are contrasting reports on the relationships between media conditions, rates of germination and virulence of conidia. It is noteworthy that these earlier evaluations were limited to single temperature and further modulation of conidia qualities by temperature interactions was not examined. Currently, there is no report on the ability of entomopathogenic fungi to assimilate solutes from growth media at different temperatures and the relationship of this with conidia virulence. However, there are possibilities that growing fungus would accumulate intracellular solutes at different rates when incubation temperature is varied. The current study has demonstrated that temperature relations during growth exert a profound influence on germination rate and virulence of conidia from cultures grown at different osmotic stress conditions (a_w).

Breaking of conidia dormancy and germination are two basic initial requirements in the process of infection by insect pathogenic fungi, and the speed at which these occur may influence pathogenicity and virulence [17]. Propagules with delayed germination could give a window of opportunity that is sufficient for the host to harness its defensive mechanisms and jeopardise overall biocontrol efficacy.

Unformulated conidia which were suspended in reverse osmosis (RO) water containing 0.02% Tween 80 was evaluated in the current study and overall, the unmodified propagules were better in terms of rate of germination and virulence. The process of development of fungi into commercial biopesticides usually involve inexpensive drying

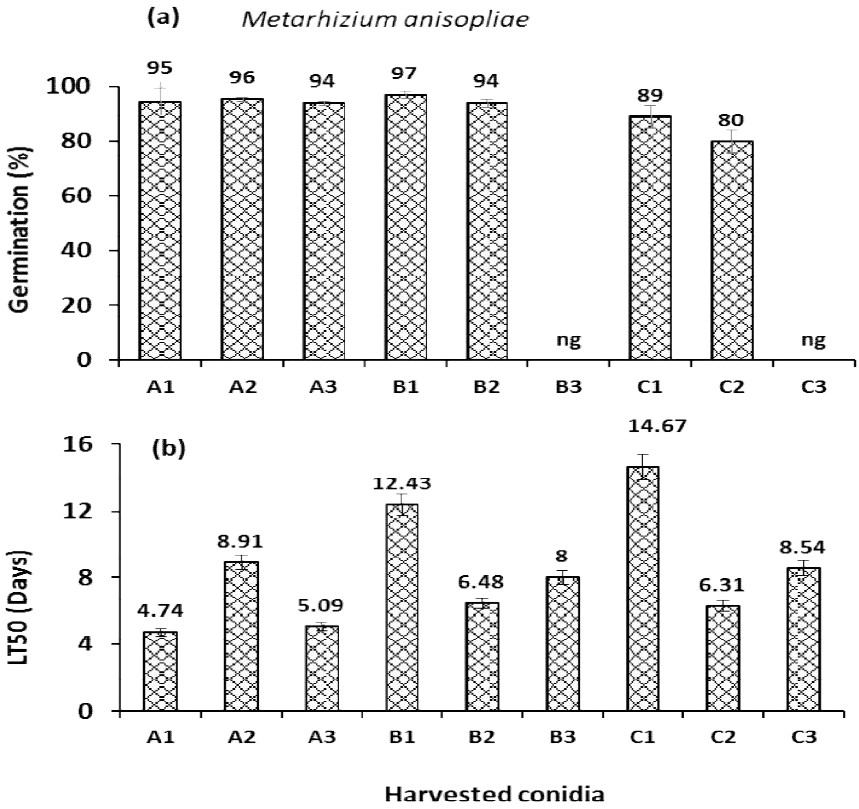

Fig. 3. Effect of media a_w and incubation temperature on quality of *M. anisopliae* conidia in relation to (a) germination rate and (b) virulence. ng =no germination

techniques to reduce production cost and conidia are often exposed to temperature treatments. Assertions from studies on the relationships between media a_w amendment and stability of conidia to dehydration and temperature stress [12,13]) suggest that this initially high relative virulence and germination capability of the unmodified conidia could be lost during formulation. To avert this, unmodified conidia can be formulated using osmoprotectants to prevent the adverse effect of drying: for example, by initially suspending conidia in skimmed milk before vacuum-drying. Although this procedure may be more expensive for commercialisation but could be worthwhile for high-quality biopesticides.

The first report on relative performance of oil-based experimental formulations of modified and unmodified conidia of *B. bassiana, I. farinosa* and *M.anisopliae* applied as a foliar spray in the field under tropical conditions for the control of whitefly, *Bemisia tabaci* showed no significant difference in biocontrol efficacy [2]. The modified and unmodified aerial conidia were initially suspended in skimmed milk and vacuum-dried before dispersing into vegetable oil. During the field experiment, ambient temperature and relative humidity often fell out of the range that was required for infection to occur, but the fungi were capable of exploiting the short favourable conditions to infect their hosts. In addition, the microclimate (relative humidity and temperature) within the plant canopy area was very conducive and within the range required by the fungi for infectivity most of the time. Production of hardy conidia as a starting material for industrial scale production of biopesticides may be unnecessary from the standpoint of biocontrol efficacy in the field.

In the current report, *B. bassiana* and *M.anisopliae* grown at the same a_w level (0.98 a_w) but incubated at different temperatures produced conidia (B1-, B2- and B3-conidia) with significantly different germination potentials and virulence. Similar variabilities also occurred in *B. bassiana* A-conidia in relation to incubation temperature. It can be suggested that temperature relations alone can be applied appropriately to improve the desirable characteristics of propagules or in addition to minor media a_w amendments during fermentation. The optimal temperature and media a_w at which the produced conidia of the isolates of *B. bassiana, I farinosa* and *M. anisopliae* showed relatively higher germination and virulence were 25°C and 0.995 a_w. These conditions are expected to vary depending on fungal isolates and strains.

6. CONCLUSION

The study has demonstrated the decisive effects of media a_w and incubation temperatures on virulence of entomopathogenic fungal conidia. It is suggested that growth conditions have to be properly defined and optimised for production of conidia before embarking on a scaled-up process. It may be possible to produce propagules that are stable (conidia with improved level of endogenous reserves) and virulent, by moderately manipulating media a_w and carefully selecting optimal interacting incubation temperature.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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