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Evaluation of Conidial Viability of Entomopathogenic Fungi as Influenced by Temperature and Additive

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Abstract

The study was conducted to evaluate the conidial viability of entomopathogenic fungi as influenced by temperature and additives. Initially five fungal isolates *i.e. Metarhizium anisopliae* (isolates- MPs, MaBg and MaCc1a), *Beauveria bassiana* (isolate- BbGc) and *Paecilomyces fumosoroseus* (isolate- PfPx) were screened by exposing conidia of each isolate to wet heat and oven heat stress through a series of temperature. Isolate MPs showed the best tolerance to the heat stress. The conidial germination of this isolate was 100%, when conidia were exposed at 30 to 35ºC temperature for all exposure intervals. Thereafter, the effect of additive was investigated on conidial viability of the isolate MPs. A total of four commonly used components and their recommended percentage used for water-dispersible granules (WG) have passed the test. Tersperse®2700 (a dispersant), 1-naphthalene sulfonic acid, sodium salt (a wetter), lignosulfonic acid, sodium salt (a dispersant-cum-binder), sodium acetate (a disintegrant), sodium alginate and sodium glutamate (as nutritive sources as well as protectant) were selected as basic components for WG-conidia formulation as they were not harmful to MPs with germination beyond 80%, when conidia were exposed to these additives. Terwet®1004 and alginic acid failed to obtain more than 80% conidial germination, hence were excluded as ingredients of WG for causing adverse effects on conidial viability. The results indicate that the conidia of this isolate might be useful as active ingredient to produce commercial WG-conidia formulation.

Keywords: Biocontrol; Formulation; Germination; Mycoinsecticide; Thermotolerance tress

Introduction

Entomopathogenic fungi are ideal for IPM programs because they are relatively safe to use and have a narrower spectrum of activity than chemical insecticides (Lacey and Goettel, 1995; Islam and Omar, 2012). It is generally perceived as providing both long-lasting insect control and having less potential for damage to the environment or non-target organisms than chemical interventions (Hokkanen and Lynch, 1995; Grace, 1997; Khetan,2001). However, they are exposed to a number of biotic and abiotic factors. These include the temperature and additive.

Temperature is the major factor that influences conidial longevity and biological activity of the formulation. In addition, the shelf life of biopesticidal product may be stored is universally dependent on temperature (Magalhães and Boucias, 2004). In general, optimum temperatures for germination, growth, sporulation and virulence of entomopathogenic fungi have been reported to range between 20 and 30ºC. Since variation in temperature tolerance among isolates can be significant **(**Ekesi, 1999; Kiewnick, 2006). Conidial thermotolerance of fungal biocontrol agents can be measured by exposing conidia to a given heat stress for a period of time and then examining their viability, which is usually represented by germination rate relative to unstressed counterparts at optimal temperature for the fungal species (Rangel *et al.*, 2004; 2005; Ying and Feng, 2004). As a wide range of field temperatures beyond the upper thermal limits of fungal growth are stressful, the efficiency and accuracy of a thermotolerance bioassay may depend mainly upon the choice of a stressful temperature that determines a physiologically tolerable period of the fungal agents and of a sampling interval over stress time (Li and Feng, 2009).

Entomopathogenic fungi, such as *B. bassiana* and *M. anisopliae*, are fungal biocontrol agents that have been formulated for wide application to insect control (Faria and Wraight, 2001; Lomer *et al.*, 2001; Roberts and St Leger, 2004; Li and Feng, 2009). Active ingredients of mycoinsecticide-sprayed conidia are sensitive to environmental stresses, such as summer temperature and UV radiation (Rangel *et al.*, 2004, 2005; Ying and Feng,

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2004); thus, the persistency and performance of a fungal formulation in the field depend, to a large extent, upon conidial tolerance to stress factors. A research activity in bioinsecticidal formulation embodies vast scientific approaches. It is a multi-disciplinary, where comprehension in microbial-plant ecology and pathology is required to elucidate the interactions amongst the biopesticide, pest and environment (Whipps, 1997; Butt *et al*., 2001). The active ingredient must be formulated with adjuvants (additives, surfactant, etc.) so that the biopesticides can be effectively delivered to the target pests. Since beneficial organisms are regarded as environmentally friendly, additives should also be environmentally benign in order to retain this advantage (Burges, 1998; Jones and Burges, 1998).

Gauging conidial sensitivity toward heat is important, because subsequent WG formulation process requires conidia to withstand beyond optimum temperature and in order to predict persistency of conidial survivability in the field and tropical weather. This study sought to determine possible types of conidial thermotolerance of 1 *B. bassiana*, 1 *P.fumosoroseus* and 3 *M. anisopliae* isolates under the thermal stresses of 30 to 50ºC for up to 60 h. The effect of different additive concerning temperature on conidial viability of entomopathogenic fungus, *M.anisopliae* (isolate MPs) was also investigated.

Materials and Methods

Entomopathogenic Fungi

Entomopathogenic fungi isolates obtained from the Faculty of Forestry, Universiti Putra Malaysia, UPM (Table 1). The isolates were cultured on SDA medium. Conidia of isolates were collected from 15-days-old cultures (maintained at 25±1ºC, 70±10% RH and L12:D12 photoperiod) and were suspended in water with 0.02% Tween 80. The conidia were quantified using a hemocytometer and a light microscope. The required fungal suspension (conidia/mL) was adjusted with regards to conidial viability (Goettel and Inglis, 1997; Islam *et al.*, 2010).

Effect of Temperature on Conidial Viability of Entomopathogenic Fungi

Aerial conidia of isolates were harvested and suspended in sterile 0.02% Tween 80. The suspension was vortexed and filtered before adjusted to a final concentration of 1×10^6 conidia/mL. An aliquot of 200 µL of the conidial suspension was added into test tubes containing 800 µL of 0.05% Tween 80. The test tubes were then exposed to wet heat in a hot water-shaker bath at six temperatures- 30, 35, 40, 42, 45 and 50°C. For each temperature, the conidial suspension was exposed to 15, 30, 45 and 60 min intervals. After exposure, the test tubes were removed from the shaker bath and submerged in icy water until ambient temperature was reached. About 150 µL of the conidial suspension was inoculated and spread onto the surface of media, sealed with Parafilm and incubated at 27±1°C for 24 h in the dark. Conidial viability was determined in terms of germination (%). Three separate fields were observed for germination at 40 × magnifications for each treatment and 100 conidia were observed randomly in each field (Islam *et al.*, 2010). Conidia with germ tubes equal to or greater than the width were considered to have germinated. Germination (%) was plotted against temperature (ºC) for each isolates in separate graphs to illustrate their respective temperature profile.

Exposure of Conidia to Oven Heat

The same procedure described above was used for preparing conidial suspension. About 150 µL of conidial suspension was inoculated and spread evenly onto the surface of the media with an L-shape rod. The Petri dishes were not sealed with Parafilm; they were incubated in oven at temperatures of 30, 35, 40, 45 and 50ºC for 24 h. After incubation, the plates were added with 0.5% of formaldehyde to inhibit further growth. Conidial viability was determined in terms of germination (%) as previously described.

Effect of Additive on Conidial Viability of Entomopathogenic Fungi

Conidia of isolate were suspended (1×10^7 conidia/mL) to 0.05% (v/v) at Tween 80 and one part of the suspension was then added to the four parts of additives to get a final concentration as recommended by the literatures (Table 2). The suspension was vigorously vortexed to break up aggregated conidia and then filtered with several layers of cheese cloth to remove mycelia mats and debris. After incubating in a water bath at 30°C for 1h, 150 µL of the conidial suspension was inoculated onto a Petri dish containing Czapek Solution Agar, CSA (Difco Laboratories, Sparks, MD, USA) and spread using a bend glass rod. The Petri dishes were incubated as similar environmental conditions as temperature effect. Conidial viability was determined in terms of germination (%) as previously described. Greater than 80% germination was indicated that the additive has passed the sensitivity test; and it was used as ingredient or excipient for WG formulation.

Protective Effects of Additives on Conidia upon Exposure to Heat

Six additives were assessed for their protective effects as mentioned in the Table 3. Firstly, the conidia were suspended in 0.05% (v/v) Tween 80 to a concentration of 1×10^7 conidia/mL. One part of the conidial suspension was then added to the four parts of additive; and diluted to the respective additive's to get a final concentration as stated in Table 3. Conidia suspended in only Tween 80 without additives served as control. The test tube was exposed to wet heat in a hot water shaker bath at 35, 40, 45, 47 and 50ºC. For each temperature, the conidial suspension wasexposedfordurationof15, 30, 45 and 60 min. Three test

Table 1: Species and the respective isolates of hypocreales fungi, original host and geographical origin

Species	Isolates	Host	Geographical origin
Metarhizium anisopliae	MaCc1a	Subterranean termite,*Coptotermes curvignathus* Holmgr.	Malaysia
	MPs	Striped flea beetle, *Phyllotreta striolata* L.	Malaysia
	MaBg	German cockroach, *Blattella germanica* L.	Malaysia
Beauveria bassiana	BbGc1	Pascoe *Gleneacelia* (Coleoptera: Cerambycidae)	Malaysia
Paecilomyces fumosorosea	PfPx1	Diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae)	Malaysia

Table 2: Additives and their concentration as per recommended by literatures used in testing the sensitivity of MPs conidia

Trade name or chemical name	Manufacturer/Supplier	Function	Final concentration of additive % (w/v)	*Literatures
Tersperse® 2700	Huntsman Corp.	Dispersant	5.0	PDS
2.Terwet® 1004	Huntsman Corp.	Wetter	5.0	PDS
1-naphthalene sulfonic acid, sodium salt or sodium naphthalene sulfonate	Sigma-Aldrich	Wetter	2.0	Morales *et al.* (1998)
Lignosulfonic acid, sodium salt or sodium lignosulfonate	Sigma- Aldrich	Dispersant/binder	12.0	Morales *et al.* (1998)
Sodium acetate	Sigma- Aldrich	Additive (buffer)	0.5	Bell *et al.* (1998)
L-glutamic acid sodium salt or sodium glutamate	BHC. Chemicals Ltd. Poole, England	Additive (nutrition)	3.0	Morales *et al.* (1998)
Alginic acid, sodium salt from brown algae or sodium alginate	Sigma- Aldrich	Additive (binder, nutrition)	0.5	Morales *et al.* (1998)
Alginic acid	Sigma- Aldrich	Additive (binder, nutrition)	0.5	N/A

*Concentrations selected for the additives were recommended by literature(s).PDS- Product Data Sheet

Table 3: Additives and their concentrations to be investigated for their protective abilities on conidia of MPs

Additives/potential protectants	Manufacturer/Supplier	*Final concentration of additive
Tween-80 (control)	Sigma Aldrich	0.05% (v/v)
Glycerol	Sigma Aldrich	5.0% (v/v)
L-glutamic acid sodium salt or sodium glutamate	BHC Chemicals Ltd. Poole, England	0.5% (w/v)
Alginic acid sodium salt from brown algae (Sigma) or sodium alginate	Sigma Aldrich	0.5% (w/v)
Lignosulfonic acid, sodium salt or sodium lignosulfonate	Sigma Aldrich	2.0% (w/v)
Sodium acetate	Sigma Aldrich	0.5% (w/v)

*Concentration was selected based on recommendation by literature(s)

tubes were prescribed for each exposure interval, for a particular temperature and additive. After exposure, the test tubes were removed from the shaker bath and submerged in ice water bath until ambient temperature was reached. The conidial inoculation was done as previously described. Conidial viability with or without protectant was then examined by determining their germination after 24 h of incubation.

Statistical Analyses

The experiments were set in a complete randomized design with three replicates for each treatment. The whole study was repeated twice with freshly prepared fungal suspension. In case of wet heat exposure, data on conidial viability were subjected to 2-way ANOVA between temperature and exposure interval under each isolate. Data regarding oven heat exposure, conidial viability were also subjected to 2-way ANOVA between temperature and isolates. Data on conidial viability to WG ingredients of *M. anisopliae* were analyzed with a one-way ANOVA. Data on conidial viability to different exposure time were subjected to 2-way ANOVA between additive and exposure time under each temperature. Conidial germination (%) was transformed using arcsine transformation to improve homogeneity of variance before analysis. The statistical analyses were performed using the Proc GLM procedure (SAS Institute, 2001). Means were separated using Tukey's Honestly Significant Different (HSD) test at 5% level of significance.

Results

Effect of Temperature on Conidial Viability of Entomopathogenic Fungi

All the isolates of *M. anisopliae* performed significantly better than the isolates of *P. fumosoroseus* and *B. bassiana*. The isolates MaCc1a and MPs were both significantly more tolerant to heat stress compared with the isolates MaBg, PfPx1 and BbGc1 for all exposure intervals (Fig. 1). The isolate PfPx1 could tolerate heating at 40ºC for 15 min with 78.7% germination (Fig. 1A). The conidial viability of PfPx1 decreased to 74% at 35 and 40ºC for 30 min exposure interval (Fig. 1A). The highest conidial germination of BbGc1 and MaBg were 66.3% and 93.3%, respectively, when conidia were exposed at 30ºC for 15 min (Fig. 1B,

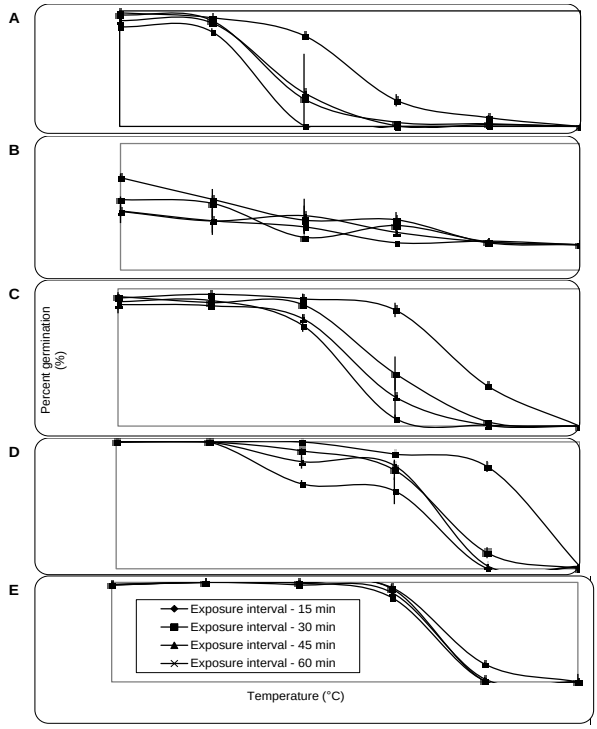

Fig. 1: Effect of heat exposure on conidial viability of entomopathogenic fungi isolatess- (A) PfPx1, (B) BbGc1, (C) MaBg, (D) MPs and (E) MaCc1a. Data were subjected to 2-way ANOVA between temperature and exposure interval under each isolate (HSD-test, $p<0.05$)

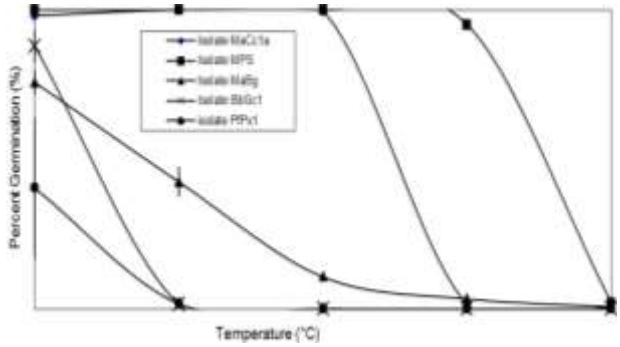

Fig. 2: Effect of oven heat exposure on conidial viability of entomopathogenic fungi after 24h exposure. Data were subjected to 2-way ANOVA between temperature and isolates (HSD-test, $p<0.05$).

1C). For 15 min exposure at 35ºC and 40ºC, the conidial viability of BbGc1 declined to 44.9% and 24.3%, respectively (Fig. 1B). A fluctuation of conidial viability of BbGc1 was recorded for throughout the exposure intervals of 30 to 60 min from 35 to 45ºC (Fig. 1B). The conidial viability of MaBg decreased to 56.7, 72.8 and 92.3% for 30 to 60 min exposure intervals, respectively at 40 to 42°C (Fig. 1C). Only 29.3% conidial germination of MaBg was recorded at 45ºC with 15 min exposure interval (Fig. 1C). The highest conidial germination with 100% achievement was observed on MPs, when conidia were exposed at 30 to 35ºC for all exposure intervals (Fig. 1D). The conidial germination of MPs was 7% and 33% for 30 and 60 min exposure intervals, respectively (Fig. 1D). The germination of this isolate continued to decline with an average rate of 9.4% for all exposure intervals at 45°C (Fig. 1D). The results clearly indicate that exposure to wet heat of isolates MaCc1a and MPs should not extend beyond 15 min to maintain acceptable viability (Fig. 1D, E).

Effect of Oven Heat Exposure on Conidial Viability of Entomopathogenic Fungi

Fig. 2 showed conidial viability of five isolates when subjected to oven heat between temperatures of 30 to 50ºC. At 30°C, the conidial viability of the isolates MaCc1a, MPs, MaBg, and BbGc1 were 98.1, 100, 75.7 and 87.8%, respectively, while the conidial viability of the isolate PfPx1 was only 40.4% (Fig. 2). When temperature was raised to 35°C, the conidial viability of both of the isolates BbGc1 and PfPx1, an acute drop of 1.5 and 1.7%, respectively, was registered (Fig. 2). At 40ºC of oven-heating, the isolates MaCc1a and MPs still maintained their viability at a germination of 99.5 and 100%, respectively (Fig. 2). Isolate MPs showed the best tolerance to the heat stress and was selected as the active ingredient in all WG-conidia formulation.

Effect of Additive on Conidial Viability of Entomopathogenic Fungi

A total of four commonly used components and their recommended percentage used for WG have passed the test. Two chemicals failed the test as germination was below80%. Tersperse®2700 (a dispersant), 1-naphthalene sulfonic acid, sodium salt (a wetter), lignosulfonic acid, sodium salt (a dispersant-cum-binder), sodium acetate (a disintegrant), sodium alginate and sodium glutamate (as nutritive sources as well as protectant) were selected as basic components of a WG-conidia formulation as they were not harmful to MPs with germination beyond 80%, when conidia was exposed to these additives (Table 4).

Terwet®1004 and alginic acid failed to obtain more than 80% conidial germination; hence they were excluded as ingredients for WG formulation due to adverse effects on conidial viability (Table 4). However, Terwet®1004 wetter at a concentration of less than 0.01% (w/w) did cause adverse effects to viability of conidia (Table 4). Tersperse® 2700 at 0.01%, the concentration was too low to have any wetting effects on the WG-formulation (Table 4).

Protective Effects of Additives from Heat Exposure

All the additives did not show any protective effects for *M. anisopliae*conidia at 35°C, because germination exposed to all the additives were not significantly higher than control

Table 4: Response of mps exposed to additives based on conidial germination

Additive	Concentration	% Germination	Germination fail or pass
Tersperse® 2700	5.0%	95.00 ± 0.88 a	pass
Terwet® 1004	5.0%	0.00	fail
1-naphthalene sulfonic acid, sodium salt	2.0%	98.44 ± 0.11 a	pass
lignosulfonic acid, sodium salt	12.0%	96.56 ± 0.59 a	pass
alginic acid	0.5%	63.89 ± 4.78 b	fail
sodium acetate	0.5%	99.00 ± 0.58 a	pass
L-glutamic acid sodium salt	3.0%	87.87 ± 12.13 ab	pass
Alginic acid sodium salt from brown algae	0.5%	93.94 ± 21.21 a	pass

Table 5: Interaction effects of additives and exposure interval on conidial viability of MPs under different temperature

Additive	Temperature (°C)	Exposure interval (Min)			
		15	30	45	60
Tween 80 (Control)	35	97.0 ± 2.1 a	90.9 ± 1.1 a	95.0 ± 0.5 a	88.8 ± 5.9 a
Glycerol		97.3 ± 1.8 a	96.0 ± 2.3 a	95.0 ± 1.0 a	93.7 ± 4.9 a
Sodium lignosulfonate		99.7 ± 0.3 a	93.8 ± 2.1 a	98.3 ± 1.7 a	84.3 ± 10.9 a
Sodium glutamate		99.0 ± 0.6 a	97.3 ± 1.8 a	98.7 ± 0.7 a	96.7 ± 1.2 a
Sodium alginate		97.3 ± 1.5 a	97.3 ± 0.9 a	97.3 ± 0.9 a	95.0 ± 2.1 a
Sodium acetate		97.3 ± 1.5 a	96.0 ± 3.1 a	93.0 ± 3.2 a	90.7 ± 2.2 a
Tween 80 (Control)	40	99.0 ± 0.6 ab	93.0 ± 4.4 ab	87.0 ± 3.5 bc	57.7 ± 6.9 b
Glycerol		99.7 ± 0.3 a	99.7 ± 0.3 a	96.3 ± 2.7 ab	94.3 ± 3.5 a
Sodium lignosulfonate		95.7 ± 0.7 b	75.5 ± 5.5 c	79.0 ± 9.0 cd	19.3 ± 3.8 c
Sodium glutamate		98.7 ± 0.3 ab	98.0 ± 1.0 a	98.0 ± 1.2 a	98.7 ± 0.7 a
Sodium alginate		97.3 ± 1.7 ab	94.3 ± 1.5 ab	93.7 ± 0.7 abc	90.7 ± 2.4 a
Sodium acetate		98.3 ± 1.7 ab	86.7 ± 1.7 bc	67.7 ± 8.7 d	23.7 ± 5.8 c
Tween 80 (Control)	45	90.5 ± 0.5 a	78.0 ± 8.0 a	81.5 ± 3.5 a	61.5 ± 10.5 a
Glycerol		17.0 ± 3.2 b	6.3 ± 2.7 c	2.3 ± 1.8 d	0.0 ± 0.0 c
Sodium lignosulfonate		88.8 ± 4.8 a	81.0 ± 3.5 a	19.7 ± 2.4 c	0.3 ± 0.3 c
Sodium glutamate		87.6 ± 3.8 a	55.3 ± 11.5 b	25.4 ± 9.6 c	13.0 ± 5.5 b
Sodium alginate		86.4 ± 1.2 a	40.0 ± 0.6 b	23.7 ± 0.3 c	8.1 ± 6.7 bc
Sodium acetate		90.3 ± 1.4 a	79.9 ± 5.4 a	52.2 ± 7.4 b	2.3 ± 1.5 bc
Tween 80 (Control)	47	36.0 ± 5.2 a	0.3 ± 0.3 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Glycerol		2.0 ± 1.5 b	0.3 ± 0.3 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Sodium lignosulfonate		2.0 ± 0.6 b	1.7 ± 1.2 a	1.7 ± 1.7 a	0.0 ± 0.0 a
Sodium glutamate		2.3 ± 0.3 b	0.3 ± 0.3 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Sodium alginate		3.3 ± 0.7 b	1.0 ± 0.0 a	0.7 ± 0.7 a	0.0 ± 0.0 a
Sodium acetate		3.3 ± 1.5 b	1.3 ± 0.3 a	0.0 ± 0.0 a	0.7 ± 0.7 a
Tween 80 (Control)	50	0.0 ± 0.0 b	0.7 ± 0.7 ab	0.7 ± 0.7 a	0.3 ± 0.3 b
Glycerol		0.7 ± 0.7 ab	0.0 ± 0.0 b	0.0 ± 0.0 a	0.0 ± 0.0 b
Sodium lignosulfonate		2.7 ± 0.3 a	2.0 ± 1.0 ab	2.0 ± 1.5 a	5.3 ± 3.0 a
Sodium glutamate		1.3 ± 0.9 ab	0.3 ± 0.3 b	0.5 ± 0.5 a	0.0 ± 0.0 b
Sodium alginate		3.3 ± 1.8 a	2.7 ± 0.7 a	1.3 ± 1.3 a	2.7 ± 1.8 ab
Sodium acetate		0.3 ± 0.3 ab	0.3 ± 0.3 b	0.7 ± 0.7 a	0.0 ± 0.0 b

Means with the same letter within a column under each temperature are not significantly different. Data were subjected to 2-way ANOVA between additive and exposure interval under each temperature followed by HSD test ($P < 0.05$) for mean comparison

(Table 5). At 40°C, germination of conidia in sodium lignosulfonate and sodium acetate was significantly lower than the other additives for exposure beyond 30 min (Table 5). Both the additives did not show any protective effects as conidial germination suspended in them and they did not significantly improve compared with control (Table 5). On the other hand, at 40°C, glycerol, sodium glutamate and sodium alginate showed significant protective effects on conidia compared with control (Table 5). At 45°C for 30 min exposure, conidia in sodium lignosulfonate and sodium acetate survived with germination of 81.0% and 79.9%, respectively (Table 5). At 45°C, conidia suspended in glycerol were adversely affecting viability with germination of 17.0% for 15 min exposure (Table 5). Beyond 45 min of exposure, all additives did not show protective effects on conidia as germination was very low (Table 5). At 60 min exposure interval, all additives failed to provide any protective effect on conidia with germination of 61.5%. At temperatures of 47°C and 50°C, germination was very low and therefore negligible (Table 5).

Discussion

Temperature tolerance becomes the most important constraint for processing by commonly used drying methods (Horaczek and Viernstein, 2004). Previous assays of conidial thermotolerance were performed at different temperatures, e.g. at 48ºC for *B. bassiana* and *P. fumosoroseus* (Ying and Feng, 2004) and at 40 or 45ºC for *M. anisopliae* (Rangel *et al.*, 2005). Conidial tolerances of

the 18 *M. anisopliae* isolates to the thermal stress of 48ºC were well represented by their LT_{50}s (10–150 min) (Li and Feng, 2009), which were spanned much more widely than those from the isolates of *B. bassiana* (10.1–61.9 min) or *P. fumosoroseus* (2.8- 6.2 min) under the same thermal stress (Ying and Feng, 2004). Conidial thermotolerance of fungal biocontrol agents varies greatly among natural strains (Rangel *et al.*, 2004; 2005; Ying and Feng, 2004; Li and Feng, 2009). Also, the conditions under which the conidia are produced can seriously affect thermotolerance (Ying and Feng, 2006). High temperatures retarded the conidial germination process in *B. bassiana* (Burges, 1998; Horaczek and Viernstein, 2004). However, the isolate used in this experiment has a good potentiality to be formulated as WG with processing temperatures not (Devi *et al.*, 2005) and *M. anisopliae* (Rangel *et al.*, 2005; Fernandes *et al.*, 2008).

The isolates of *M. anisopliae* were more heat tolerant compared with *B. bassiana*and *P. fumosoroseus* in both forms of heating regimes (Figs. 1 and 2). A similar phenomenon was reported by Horaczek and Viernstein (2004) that *M. aniospliae* isolate was slightly better than *B. bassiana*. A heat-resistant test by revealed that *M. anisopliae*did not lose viability after being exposed to 40 and 45°C for 15 min (Horaczek and Viernstein, 2004). However, it was found that *B. bassiana*exposed for 15 min at 40 and 45°C, resulted in a 17 and 43% reduction in activity, respectively. A study to evaluate conidial thermotolerance of *M. anisopliae* isolates from difference geographical origins recorded three isolates that could tolerate temperature between 41-49°C for as long as 4 h of exposure (Rangel *et al.*, 2005). Many reports showed that thermotolerance or heat resistance characteristics of conidia were correlated with its geographical origin (Selman *et al.*, 1997; Vidal *et al.*, 1997). The variability of thermotolerance amongst entomopathogenic isolates underlines the importance of studying interaction between pathogen/host/environment factors in order to select potential candidate(s) for biological control (Ekesi *et al.*, 1999). Our experiments showed that the isolate MPs was selected as the active ingredient for all subsequent WG-conidia formulation as it was the most tolerant to high temperature of both oven and wet heat exposures compared with other isolates. No exposure to any exothermic process should be allowed beyond 15 min.

The isolate MPs was sensitive to several ingredients for typical WG-conidia formulation. Terwet®1004 was rejected as an ingredient, although it is a common industrial wetter that is used for conventional WG formulation. However, Terwet®1004 at a concentration of less than 0.01% (w/w) did not cause adverse effects to the viability of conidia with germination of 94%, but this concentration was below the recommended wetter concentration range in conventional WG-formulations (Suzuki, 2008). The wetting function was replaced by 1-naphthalene-sulfonic acid, sodium salt which is also common in conventional WG formulations that falls well within the recommended concentration of between 1-2% (w/w) (Suzuki, 2008). Alginic acid, at recommended rate of 0.5% aqueous solution has a pH of 3.0 ± 0.5 and this high acidity may have adverse effects on the viability of conidia (Fig. 3). A similar phenomenon was reported (Potyka, 1996) that alginic acid at 0.1% (w/v) was shown to inhibit germination of *Colletotrichum dematium*, a myco-herbicide. The sodium salt of alginic acid was used instead and was found to have slight protective properties with respect to conidia exposure to high temperatures in our experiments especially by prolonging viability for 30 min at 40°C (Fig. 3). Sodium alginate is a common excipient in control-released formulation by forming hydrogels matrix with calcium ions with biological propagules (Kikuchi *et al.*, 1997), but its incorporation as additive and its role in WG has not been clarify.

To conclude, our investigation on the potential of additives used in WG formulation as protectants showed that sodium alginate, sodium glutamate and glycerol have protective effects on conidia with significantly higher germination compared with control for 60 min exposure interval at 40°C (Table 5). When conidia suspended in sodium lignosulfonate and sodium acetate had slight protective effects on conidial viability compared with other additives for 30 min exposure interval at 45°C (Table 5). Incorporation of protective additives in formulation, the conidia showed protective effects during heat treatment exceeding 47ºC for less than 15 min exposure interval. This isolate might be useful as active ingredient to produce commercial WG-conidia formulations.

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