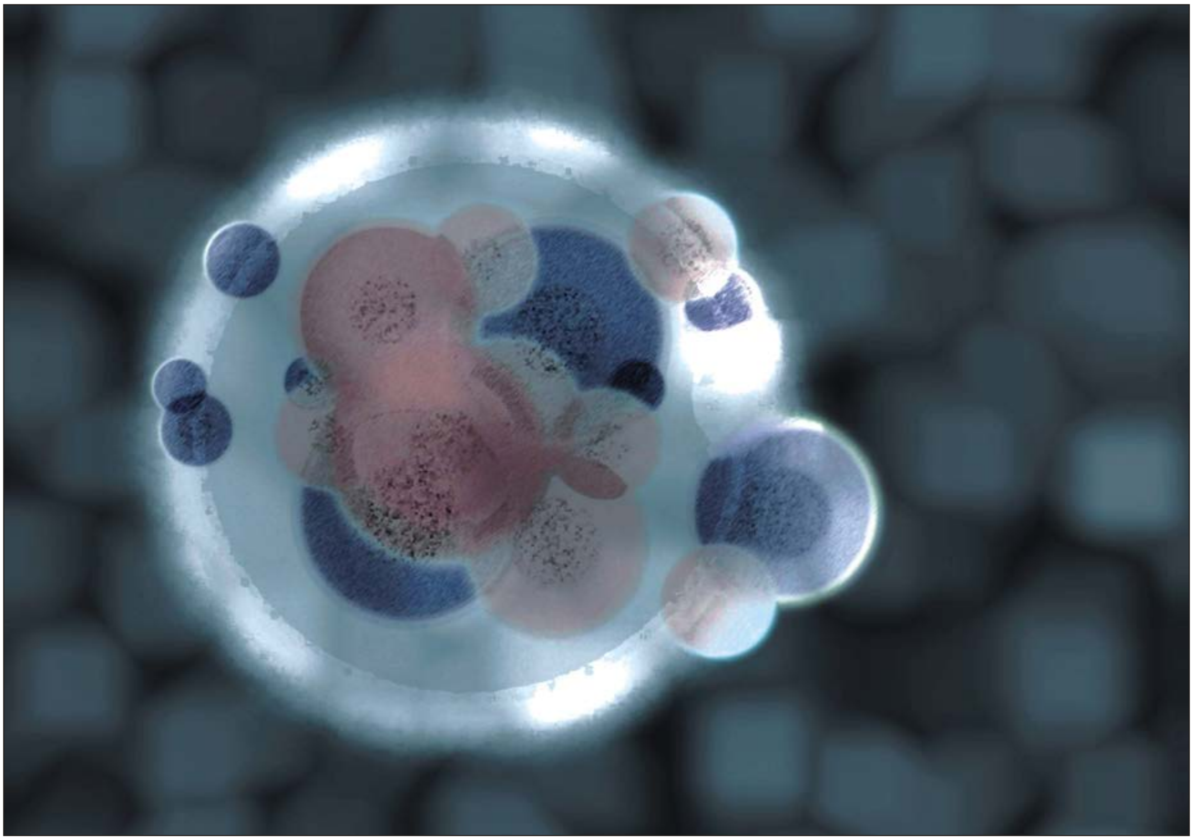

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Comparison of House Fly Forewing and Artificial Media for Examining Environmental Effects on Conidial Germination of Entomopathogenic Fungi

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Abstract: The objectives of this study were to test the use of a forewing of the house fly (*Musca domestica* L.), ¼ strength sabauroud dextrose agar (SDAY/4) and dry agar-agar as substrates to assess the effect of relative humidity (RH) (91, 95, 98 and 100±1%) and temperature (15, 20 and 30±1°C) on spore germination of *Beauveria bassiana* 5672 and *Metarhizium anisopliae* 1080 isolates at 24, 48 and 72 h post exposure. In all treatments tested, similar influences of temperature and %RH were observed when conidial germination of 5672 and 1080 was tested on SDAY/4 and fly forewing, whereas little or no germination occurred with agar-agar as a substrate. Conidial germination occurred at temperatures of 15-30°C and ≥95% RH and temperatures of 20-30°C and >95% RH for *B. bassiana* 5672 and *M. anisoplia* 1080, respectively. At 100% RH, 30°C and 72 h exposure period, the highest germination rates of 1080 were 99 and 96% on SDAY/4 and fly forewing, respectively. We concluded that the SDAY/4 medium triggered the conidial germination of these fungi similar to the house fly forewing cuticle. This suggests that SDAY/4 medium is useful as a substitute substrate of cuticle when examining the influence of temperature and %RH on conidial germination. The SDAY/4 medium is also nutritive and supplies a substrate for growth and sporulation. This technique provides a tool for predicting the epidemiology of these fungi in the field for an IPM program of Sunn Pest.

Key words: *Beauveria bassiana, Metarhizium anisopliae*, temperature, relative humidity, germination, forewing house fly, nutrient media

INTRODUCTION

Sunn Pest, *Eurygaster integriceps* Puton, is an important pest of wheat and barley (Banks *et al*., 1961; Critchley, 1998). Up to 100% yield losses can arise if control methods are not applied (Skaf, 1996). This insect releases enzymes while feeding that degrade the gluten quality of the grains. Grains are not suitable for baking if ≥2-3% of the grain is damaged (El-Haramein *et al.,* 1984). Entomopathogenic fungi as biological control agents have been used successfully in management programs for greenhouse, field, pasture, tree fruit, forest and urban pests (Inglis *et al*., 2001). Sunn Pest spends approximately 9 mo under the litter of pine or oak trees in overwintering sites located in the hills and mountains nearby wheat and barley fields (Banks *et al*., 1961). Several entomopathogenic fungi have been isolated from Sunn Pest in overwintering sites (Parker *et al*., 2003). These may have potential for use in biological control of Sunn Pest as it overwinters.

Temperature and Relative Humidity (RH) affect germination of entomopathogenic fungi (Arthurs and Thomas, 2001; Carruthers and Hural, 1990; Sivasankaran *et al*., 1998). The effects of temperature alone at 20, 25, 30 and 35°C on growth and sporulation of 31 isolates were investigated by Parker *et al*. (2003). However, there have been no other studies examining the interaction of temperature and %RH occurring in the Sunn Pest overwintering sites on the germination of entomopathogenic fungi.

The availability of a suitable technique for investigating the interaction of temperature and %RH on conidial germination may be responsible for the apparent lack of study. The substrate conidia are in contact with, for example cuticle or nutritive media can influence germination. Germination of entomopathogenic fungi is either inhibited or triggered by cuticle components of insects. The inhibitory effects can be related to different causes. For example, James *et al*. (2003) found that the nymphal cuticular lipids of silverleaf whitefly, *Bemisia argentifolii* Bellows inhibited the germination of *Beauveria bassiana* (Balsamo) Vuillemin and *Paecilomyces fumosoroseus* (Wize) on the fourth instars of this pest. These cuticular lipids inhibit the conidial germination by acting as hydrophobic barriers that prevent the conidia from reaching the water or nutrient components of the cuticle (James *et al*., 2003). Sosa-Gomez *et al*. (1997) indicated that aldehyde (*E*)-2-decenal inhibited the conidial germination of *Metarhizium anisopliae* Metschnikov (Sorokin) (5-20%). The aldehyde (*E*)-2-decenal is a primary component of the abdominal scent gland of the cuticle of the fifth instars of green stink bugs, *Nezara viridula* (Linnaeus) (Waterhouse *et al*., 1961). Chemical components of the cuticle can also play an inhibitory role in fungal growth and development (Szafranek *et al*., 2001). The free fatty acid groups in the exoskeleton lipids of aphids, *Hyalopterus pruni* (Geoffroy), *Brevicoryne brassicae* (Linnaeus) and *Sitobion avenae* (Fabricius) were found to be responsible for inhibiting the mycelia growth and sporulation of the entomopathogenic fungi, *B. bassiana* and *P. fumosoroseus* (Szafranek *et al*., 2001).

Conidial germination can be also triggered by cuticle components. For instance, larval cuticle of *Helicoverpa zea* (Boddie) triggered the conidial germination of the entomopathogenic fungi, *Nomuraea rileyi* (Farlow), when added to yeast extract to create a minimal medium (El-Sayed *et al*., 1993a). Germination of *N. rileyi* was stimulated when cuticle of *H. zea*, *H. virescens* (Fabricius) and *Trichoplusia ni* (Hübner) where added to a solid substrate (El-Sayed *et al*., 1991). Cuticle components triggered the germination of *N. rileyi* by inducing the release of protease and chitinase enzymes that help in the penetration of the larval cuticle (El-Sayed *et al*., 1993b).

This paper is part of a study for the development of *B. bassiana* and *M. anisopliae* for Sunn Pest management. We assessed the effect of temperature and RH on spore germination of *B. bassiana* and *M. anisopliae* using the house fly forewing, quarter–strength sabauroud dextrose agar (SDAY/4) and agar-agar substrates at 24, 48 and 72 h post exposure period. These substrates were used to provide an accurate evaluation for %RH effects on germination because they are dry and transparent substrates, so no additional humidity is added to the system and conidia can be observed clearly under the microscope. The forewing of the house fly was used to compare wing cuticle with media for the germination of tested isolates. SDAY is a standard nutrient medium, however dry agar-agar is a nutrient free medium and was therefore used for comparison purposes. Investigating spore germination of these fungi under temperatures and RH levels that occur in the Sunn Pest overwintering sites will help in predicting the epidemiology of these fungi in the field. This will also help in the development of entomopathogenic fungi for Sunn Pest management in overwintering sites in West Asia.

MATERIALS AND METHODS

Germination substrate and exposure time evaluation: Fungal isolates preparation: Two fungal isolates were used in this study: *B. bassiana* ARSEF code 5672, which had promising activity against Sunn Pest in laboratory trials (Parker *et al*., 2003) and *M. anisopliae* ARSEF code 1080, originally isolated from corn earworm, *H. zea* (Boddie) in the United States (St. Leger *et al*., 1992). Each isolate is maintained at the US Department of Agriculture, Agriculture Research Service Collection of Entomopathogenic Fungi (ARSEF). A fresh culture of each isolate was established in 9 cm diam Petri dishes containing quarter–strength sabauroud dextrose agar (SDAY/4) [neopeptone 2.5 g, dextrose 10 g, yeast extract 2.5 g, agar 15 g, citric acid 0.4 mL (50 g in 100 mL dH_2O) and water 1 l] and incubated at 24±2°C for 10 day.

A stock suspension of each isolate was prepared by adding 10 mL 0.05% Tween 80 directly onto the Petri dish containing the fungal culture. The fungal materials were scraped from the medium and pipetted into a 15 mL glass test tube containing small glass balls (1-2 mm diam.). The suspension was vortexed for ≈5-10 min to separate the conidia from mycelia and produce a homogenous suspension. The suspension was filtered through eight layers of cheesecloth, after which 1 mL was pipetted into another tube containing 9 mL of Tween 80 (0.01%). This was vortexed thoroughly and diluted to achieve a suspension of 1×10^6 conidia mL^{-1} (Goettel and Inglis, 1997). Suspensions (1×10^6 conidia mL^{-1}) of each isolate were chilled on ice to prevent germination prior to exposure to the test temperature and RH.

Germination assessment: Three types of materials were tested as substrates on which to assess spore germination: the forewing of the house fly (*Musca domestica* L.), dry agar-agar (Sun Wing Hong Food LTD, Hong Kong) and dried SDAY/4 medium.

Pupae of the house fly, *M. domestica* were obtained from Beneficial Insectary Company (Oak Run, California) and held at room temperature in a glass box. Flies emerged in 1-2 days and were frozen for 2 h. Frozen flies were pinned in the thorax using an insect pin (size 2) and the forewings removed with forceps. Wings were kept in petri dishes (9 cm diam) lined with filter paper and kept in the refrigerator until used. We used dry agar-agar in our experiment as a substrate to test the germination because it is transparent allowing conidia to be viewed with a microscope and because it is dry, the %RH that conidia are exposed to will not be affected.

The spore germination tests were conducted in 6 cm diam Petri dishes. In each petri dish, 5 forewings, 5 squares of dry agar-agar (1×1 cm) and 5 drops (40 μL) of SDAY/4 media were used as germination test substrates. Petri dishes were left under sterile hood conditions for 40 min to allow the drops of the SDAY/4 media to dry. In each petri dish, 10 μL of the suspension (1×10^6 conidia mL^{-1}) were pipetted on each of the three substrates. Petri dishes were allowed to dry an additional 30 min before temperature and RH exposure. The suspension on the forewings and agar-agar squares adhered to the bottom of the dish as the suspension dried and therefore did not cross-contaminate each other during the experiment. This facilitated microscopic observation of spore germination.

Incubators were set at 15, 20 and 30±1°C. In each incubator, saturated salt solutions of barium chloride, potassium nitrate and potassium sulphate were used to provide 91, 95 and 98±1%RH (Center for microcomputer applications, 2003). Distilled water was used to provide 100±1%RH (Arthurs *et al*., 2001). The following protocol was used to maintain the %RH at each temperature.

The salt solutions or water were poured into plastic boxes (30×25×10 cm, length×width×height) to a depth of 3 cm. A platform held 1 cm above the solution was constructed from metal screening supported by corks at each corner. Petri dishes containing the three substrates were placed on the platform and held in place with metal frames attached with twist ties. After 2-6 h in an environmental chamber set to the target temperature, the test containers equilibrated to the desired temperature and humidity. Preliminary verifications of atmospheric conditions within the test containers were made during protocol development using Oregon Scientific Cable Free Remote Thermo-hygrometer THGR-268 (Oregon Instruments, Cannon Beach, OR). The above procedures were repeated for each %RH at different temperatures using the salt solution mentioned above.

After reaching the required %RH at each temperature, the Petri dishes were placed inside each box on the screen. The changes in %RH found in preliminary experiments when boxes were opened and closed to add Petri dishes. Boxes were placed inside clear plastic bags sealed with binder clips to maintain the target %RH and incubated in the dark to prevent a light effect.

Germination was tested at 24, 48 and 72 h post exposure, by having a separate Petri dish for each exposure period. The Petri dishes for each exposure period were housed in separate %RH treatment boxes to avoid fluctuations in %RH during opening of the boxes. Germination was stopped for each treatment by adding one drop of lacto phenol cotton blue on each of the 5 pieces of each substrate [(5 forewings, 5 squares of dry agar-agar (1×1 cm) and 5 drops (40 μL) of SDAY/4 media)] in each Petri dish. The substrate pieces were then covered with glass coverslips and left open to dry for 1-2 h. Petri dishes were stored at 5°C until inspection (Hywel-Jones and Gillespie, 1990). In each Petri dish, 500 conidia were counted per substrate (100 per each piece of the 5 pieces of the substrate under the coverslip) under microscope 400X and the numbers of germinated and non-germinated conidia were counted. Conidia were scored as germinated if the germ-tube was equal to or longer than the conidial width (Arthurs and Thomas, 2001; Dillon and Charnley, 1990). Values for the five sub-samples per substrate in a Petri dish were averaged to obtain the estimated percent germination. The experiment was repeated three times.

Data analysis: The experimental design was a factorial design with five factor levels (isolates, temperature, RH, substrates and time post-exposure). Three runs were made for each experiment with one replication of each run being based on the sub-sample averages. SAS PROC GLM (SAS Institute, 2002) was used for data analysis. Up to three way interactions were evaluated at $\alpha = 0.05$.

RESULTS

We found that temperature, %RH, substrate, isolate and post exposure period influenced the conidial germination of *B. bassiana* 5672 and *M. anisoplia* 1080 (Table 1). In general, similar influences of temperature and %RH were observed when conidial germination of 5672 and 1080 was tested using SDAY/4 and fly forewing as substrates, whereas little or no germination occurred with agar-agar as a substrate. A variety of higher order interactions occurred. In particular, temperature interacted significantly with each of the other main effects, either individually or in combination with other main effects. In most cases, there was a notable lack of interaction between %RH and the other main effects, except temperature (Table 1). At ≥98% RH, differences in conidial germination at temperatures tested for SDAY/4 and house fly forewing were obvious at 24 h exposure period, but germination trends became almost identical at ≥48 h exposure period (Fig. 2 and 3).

No germination occurred for entomopathogenic fungi tested at 91% RH for all temperatures, germination substrates and exposure periods. At 95% RH and with all other test parameters, no germination occurred for *M. anisoplia* isolate 1080. However, for *B. bassiana* isolate 5672, low germination (up to 30.5 and 30%) occurred only at 20°C on SDAY/4 and fly forewing, respectively (Fig. 1). At 95% RH and 20°C, conidial

Table 1: Analysis of variance for conidial germination of *B. bassiana* (5672) and *M. anisopliae* (1080) isolates on a forewing of the house fly, dried SDAY/4 and dry agar-agar at RH (98 and 100±1%) and temperatures (15, 20 and 30±1°C) at 24, 48 and 72 h post exposure. Data from 91 and 95% RH not included because little or no germination was detected

Source	DF	Type III SS	Mean square	F-value	p>F
Run	2	187.78	93.89	0.65	0.5245
Temperature	2	28911.23	14455.62	99.62	<.0001
Post exposure period	2	112885.57	56442.79	388.98	<.0001
%RH	1	3848.50	3848.50	26.52	<.0001
Isolate	1	31820.88	31820.88	219.30	<.0001
Substrate	2	241662.99	120831.49	832.73	<.0001
Temperature *%RH	2	3114.05	1557.03	10.73	<.0001
Temperature *Isolate	2	5282.58	2641.29	18.20	<.0001
Temperature *Post exposure period	4	2991.50	747.87	5.15	0.0005
Temperature *Substrate	4	12659.55	3164.89	21.81	<.0001
%RH*Isolate	1	199.72	199.72	1.38	0.2418
Post exposure period*%RH	2	468.79	234.39	1.62	0.2009
%RH*Substrate	2	537.98	268.99	1.85	0.1588
Post exposure period*Isolate	2	4572.62	2286.31	15.76	<.0001
Isolate*substrate	2	13621.93	6810.96	46.94	<.0001
post exposure period*Substrate	4	56457.55	14114.39	97.27	<.0001
Temperature *%RH*Isolate	2	1077.15	538.57	3.71	0.0258
Temperature *Post exposure period* %RH	4	2878.43	719.61	4.96	0.0007
Temperature *%RH*Substrate	4	1681.10	420.27	2.90	0.0227
Temperature *Post exposure period*Isolate	4	7088.55	1772.14	12.21	<.0001
Temperature *Isolate*Substrate	4	3276.84	819.21	5.65	0.0002
Temperature *Post exposure period*Substrate	8	7744.44	968.06	6.67	<.0001
Post exposure period * %RH * Isolate	2	1051.49	525.75	3.62	0.0281
%RH*Isolate*Substrate	2	532.43	266.22	1.83	0.1618
Post exposure*%RH*Substrate	4	685.40	171.35	1.18	0.3197
Post exposure period * Isolate * Substrate	4	6946.02	1736.50	11.97	<.0001
Error	248	35985.59	145.10		
Total	321	590948.32			

germination was 0, 7.5 and 30.5% at 24, 48 and 72 h exposure period, respectively when SDAY/4 substrate was used. Similar results were observed on fly forewings as a germination substrate with conidial germination of 0.6, 6 and 30% at 24, 48 and 72 h exposure period, respectively. Data from 91 and 95% RH were not included in analysis because no or little germination was detected.

At 98 and 100% RH, high conidial germination occurred at 48 and 72 h exposure period for *B. bassiana* isolate 5672 at all temperatures tested. At 20°C and 72 h exposure period on SDAY/4, conidial germination of *B. bassiana* isolate 5672 was 99.6 and 99.9% at 98 and 100% RH, respectively (Fig. 2 and 3). Similar results were found on fly forewings with conidial germination of 98.5 and 99.2% at 98 and 100% RH, respectively (Fig. 2 and 3). At 100% RH, 30°C and 72 h exposure period, the highest germination of *M. anisopliae* isolate 1080 occurred with 99 and 96% on SDAY/4 and fly forewing, respectively (Fig. 3).

As might be expected, longer exposure periods across all temperatures, %RH, regimes and germination substrates resulted in greater conidial germination. Germination at 72 h was slightly higher than at 48 h and generally higher than at 24 h (Fig. 1, 2 and 3). In general, the highest germination occurred at 30°C, 100% RH, 72 h exposure period and on SDAY/4 and house fly forewing as germination substrates for *B. bassiana* isolate 5672 and *M. anisopliae* isolate 1080

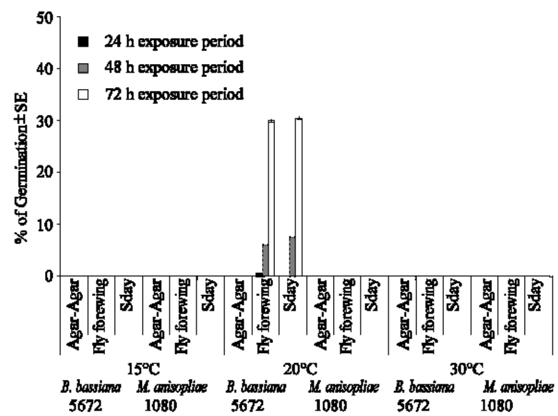

Fig. 1: Conidial germination of *B. bassiana* isolate (5672) and *M. anisopliae* isolate (1080) on dried SDAY/4 medium, dry agar-agar and forewing of the house fly at 95% RH and temperatures (15, 20 and 30±1°C) for 24, 48 and 72 h post exposure period

DISCUSSION

In present study, we found that conidial germination of *B. bassiana* (5672) and *M. anisopliae* (1080) was

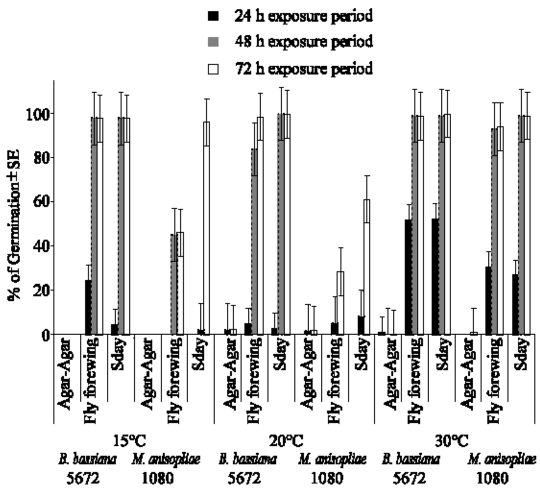

Fig. 2: Conidial germination of *B. bassiana* isolate (5672) and *M. anisopliae* isolate (1080) on dried SDAY/4 medium, dry agar-agar and forewing of the house fly at 98% RH and temperatures (15, 20 and 30±1°C) for 24, 48 and 72 h post exposure period

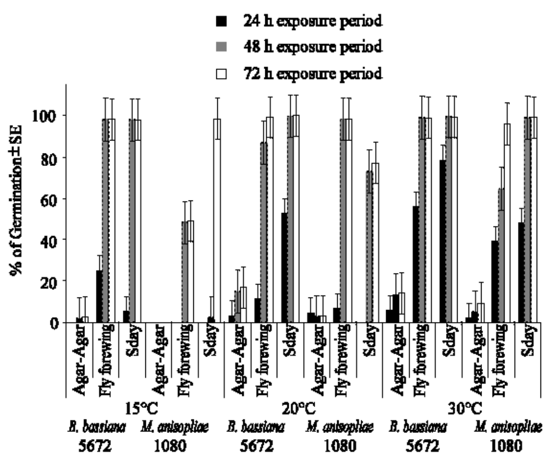

Fig. 3: Conidial germination of *B. bassiana* isolate (5672) and *M. anisopliae isolate* (1080) on dried SDAY/4 medium, dry agar-agar and forewing of the house fly at 100% RH and temperatures (15, 20 and 30±1°C) for 24, 48 and 72 h post exposure period

high on both SDAY/4 and house fly forewing at ≥98% RH, 20-30°C and 48-72 h exposure period. The body wall is similar in composition to wing cuticles (Jarrold *et al*., 1998), therefore investigating conidial germination on house fly wing cuticles provides useful information about their germination on insect cuticles. Very little has been published on wing cuticle as a substrate for conidial germination. *Metarhizium anisopliae* var. *acridum* (Metschnikoff) Sorokin germinated well on wing from a fly *Calliphora vomitoria* L. and a beetle, *Tenebrio molitor* L. (Jarrold *et al*., 1998). SDAY/4 is a nutrient medium used in fungal isolation, culture preparation and sporulation studies of entomopathogenic fungi (Lezama-Gutierrez, 1996; Magalhães *et al*., 2000). In this study, we used five drops (40 μL) of this medium in each Petri dish to allow the water content to dry out quickly leaving the nutritious components to be used by fungi. The development of this new approach allowed us to use this nutrient medium and achieve an accurate evaluation for %RH effects on germination. This is because drops of this medium were dry and transparent, so no additional humidity was added to the system and conidia were clearly observable under the microscope. *Metarhizium anisopliae* (Bidochka *et al*., 2001) and *B. bassiana* can germinate and grow saprophytically in the soil when the host insects are not available (Padmavathi *et al*., 2003). Growth on a nutritive media is more likely representative of the saprophytic rather than pathogenic growth process. However, utilization of the cuticle alone is probably representative of the germination process since entomopathogenic fungi continue growth within the insect hemocoel (MacLeod, 1963). Since the conidial germination of *B. bassiana* and *M. anisopliae* isolates tested was high on both the house fly forewings and SDAY/4 medium and little or no germination occurred on agar-agar (non-nutrient medium) at all temperatures, %RH and exposure periods tested (Fig. 1, 2 and 3), we conclude that the SDAY/4 medium triggered the conidial germination of these fungi similar to the house fly forewing cuticle.

Hart and Mcleod (1955) found that the conidial germination of *B. bassiana* occurred at temperatures between 10-28°C. While, McCammon and Rath (1994) found that the conidial germination for *M. anisopliae* occurred between a range of temperatures from 2.5-37°C. We found that germination of *B. bassiana* and *M. anisopliae* isolates on all substrates tested occurred at temperatures between 15-30°C and the highest germination rates occurred at 30°C. However, Teng (1962) found maximum germination for *B. bassiana* on pine caterpillar, *Dendrolimus punctatus* Walker was at 24°C.

Temperature and %RH combinations affect the conidial germination of entomopathogenic fungi (Glare *et al*., 1986; Liu *et al*., 1989; Steinkraus and Slaymaker, 1994). Optimal conidial germination of a strain of *M. anisopliae* var. *anisopliae* (MA-1) isolated from coconut leaf beetles, *Brontispa longissima* Gestro was observed at temperatures of 24-30°C and 92.5-100% RH (Liu *et al*., 1989). Walstad *et al*., (1970) indicated that conidial germination of *B. bassiana* and *M. anisopliae* occurred at temperatures between 15-35°C and >92% RH. The germination of the entomopathogenic fungus, *Zoophthora phalloides* Batko occurred at ≥98% RH and at relatively cold temperatures from 10-20°C (Glare *et al.*, 1986). Spore germination for most entomopathogenic fungi require at least 95% RH at the insect surface (Hallsworth and Magan, 1999). In this study, we found that *B. bassiana* and *M. anisopliae* isolates germinated at temperature of 15-30°C and ≥95% RH and temperature of 20-30°C and >95% RH for *B. bassiana* and *M. anisopliae*, respectively. The lower thermal tolerance of the isolate of *B. bassiana* might make it more suitable for use in overwintering sites. At >95% RH, high conidial germination was observed at 30°C on either SDAY/4 or house fly forewing substrates for both fungi tested.

We found that %RH positively influenced the germination of entomopathogenic fungi tested. In all cases, a significant three-way interaction ($p \leq 0.05$) was observed between %RH and temperature regimes tested (Table 1). However, in our study, no significant three-way interactions ($p > 0.05$) were found between %RH and isolates, post exposure periods and substrates tested (Table 1); whereas there was a significant three-way interaction ($p \leq 0.05$) between temperature and these factors (Table 1). Therefore, the effect of %RH on conidial germination is not dependent on isolates and substrates tested. Maximum effects of entomopathogenic fungi require a high %RH level at an early stage of infestation. For example, at 75% RH for a 1 min exposure period, only 29.4% of conidia of *Neozygites fresenii* (Nowakowski) formed capilliconidia when later exposed to 100% RH, whereas 76.2% formed capilliconidia when exposed continuously to 100% RH (Steinkraus and Slaymaker, 1994). Similarly, germination of *B. bassiana* did not occur at a RH of 75-85% at all temperatures tested (Jurianto and Sri, 1995). We conclude that %RH plays a more significant effect on fungal germination than temperature.

In conclusion, the house fly and SDAY/4 triggered conidial germination similarly at most %RH and temperatures tested. We also suggest that SDAY/4 medium is a useful substrate for investigating fungal germination to help predict the epidemiology of these fungi in the field. Moreover, because house fly forewing and SDAY/4 medium are dry and clear substrates, they can be used in examining the effects of %RH on conidial germination of entomopathogenic fungi and conidia can be obviously distinguished under microscope. SDAY media may be better suited in general for studying spore germination because it does not appear to have inhibitory qualities sometimes found with insect cuticles. However, in cases where interactions between conidia and specific insect hosts are of interest, the use of host cuticle would be more appropriate. Present data provides useful information about the influences of temperature and %RH occurring in overwintering sites on germination of *B. bassiana* and *M. anisopliae* isolates and supports the development of an IPM program for Sunn Pest.

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