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Sprays of emulsifiable *Beauveria bassiana* formulation are ovicidal towards *Tetranychus urticae* (Acari: Tetranychidae) at various regimes of temperature and humidity

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Abstract Aerial conidia of *Beauveria bassiana* in an emulsifiable formulation germinated by >95% after 24 h exposure to the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH. Ovicidal activities of the formulation towards two-spotted spider mite, *Tetranychus urticae*, were assayed at the concentrations of 0, 18, 160 and 693 conidia mm^{-2} sprayed separately onto fava bean leaves including 39 (25–76) eggs per capita. All the sprayed eggs on the leaves were directly exposed to the different regimes for hatch after 24 h maintenance in covered Petri dishes. Generally, hatched proportions increased over post-spray days and decreased with the elevated fungal concentrations; no more eggs hatched from day 9 or 10 onwards. Based on the counts of the hatched/non-hatched eggs in the different regimes, the final egg mortalities were 15.0–40.4%, 48.9–66.6% and 62.9–87.5% at the low, medium and high concentrations, respectively, but only 5.6–11.3% in blank controls. The RH effect on the fungal action was significant at 20 and 25°C but not at 30°C whereas the effect of temperature was significant at 51% and 74% RH but not at 95% RH. Probit analysis of the egg mortalities versus the fungal sprays generated median lethal concentrations (LC_{50}) of 65–320 conidia mm^{-2} at all the regimes, and of only 65–78 conidia mm^{-2} at 25–30°C with 74–95% RH. The results highlight ovicidal activities of the emulsifiable formulation against the mite species at the tested regimes and its potential use in spider mite control.

Keywords *Beauveria bassiana* · *Tetranychus urticae* · Fungal formulation · Ovicidal activity · Environmental effect · Spider mite control

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Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch [including synonymous *T. cinnabarinus* (Boisduval); Ros and Breeuwer 2007], infests a large variety of economic plants worldwide (Hazan et al. 1974; Ho et al. 1997). Spider mite control in the past few decades has relied upon a number of acaricides, such as organochlorides and organophosphates (Gerson and Cohen 1989). This reliance on chemicals has generally caused mite resistance and public concerns on their high residues in products (Guo et al. 1998; Dagli and Tunc 2001). Some acaricides, such as dicofol, cyhexatin and fenbutatin oxide, have thus been prohibited from mite control on vegetables, melons, fruits and tea in China, making it necessary to search for alternative control measures. Fungal pathogens of mites are considered to be potential for the purpose (Poinar 1998; Chandler et al. 2000; Van der Geest et al. 2000).

Entomopathogenic hyphomycetes, such as *Beauveria bassiana* (Balsamo) Vuellemin, *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith, are well-known fungal biocontrol agents (Feng et al. 1994; Faria and Wraight 2001; Roberts and Leger 2004) and have been formulated for wide application to insect control (Langewald et al. 1997; Wraight et al. 2000; Wraight and Ramos 2002; Feng et al. 2004a, b; Pu et al. 2005). They are also potential mite pathogens despite rare prevalence in the field (Chandler et al. 2000). Recently, some fungal isolates derived from host insects have proven to kill spider mite eggs under laboratory conditions and unformulated conidia of a *B. bassiana* isolate have an ovicidal LC_{50} of 548 conidia mm^{-2} (Shi and Feng 2004), which can be reduced greatly by low application rates of pyridaben included in fungal sprays (Shi et al. 2005). In other studies, the fungal insect pathogens are also found capable of infecting active stages of spider mites (Alves et al. 2002; Wekesa et al. 2005, 2006; Maniania et al. 2008; Shi et al. 2008a) and ectoparasitic mites (Shaw et al. 2002; Lekimme et al. 2006, 2008; Meikle et al. 2008).

Environmental temperature and relative humidity (RH) are known to affect conidial germination, colony growth, and host infection of the fungal pathogens (Feng et al. 1994; Roberts and Leger 2004). Appropriate temperature and high RH are usually crucial to successful infection of the fungal agents (Milner et al. 1997; Luz and Fargues 1999). Although common fungal agents in unformulated form have proven to infect various stages of mite pests under controlled conditions (Shi and Feng 2004; Lekimme et al. 2006; Wekesa et al. 2005, 2006), the possible effects of selected formulations and variable environments on their acaricidal activities have not yet been understood. This has hindered a sound evaluation of their potential in mite control. In the present study, aerial conidia of the ovicidal *B. bassiana* isolate found previously (Shi and Feng 2004) were formulated into an oil-based, emulsifiable carrier and then sprayed onto leaves where *T. urticae* eggs were laid in advance. Our goals were to evaluate ovicidal activities of the formulation at gradient application rates and to determine the effects of different temperature and humidity regimes on the hatch rates and mortalities of the mite eggs. The data presented in this paper would help to value the potential of the fungal formulation for incorporation into mite pest management systems.

Materials and methods

Preparation of aerial conidia and emulsifiable formulation

The ovicidal isolate, *B. bassiana* SG8702, was derived from a naturally mycosed aphid (Feng et al. 1990) with accession number ARSEF 2860 (USDA-ARS Collection of

Entomopathogenic Fungal Cultures, Ithaca, NY, USA). This isolate has been formulated for control of greenhouse whiteflies (Feng et al. 2004a) and tea leafhoppers (Feng et al. 2004b; Pu et al. 2005). It was preserved as a mixture of dried conidia with sterile sands at −72°C. To produce conidia in this study, the preserved conidia were used to inoculate the plates of Sabouraud dextrose agar plus 1% yeast extract (SDAY) for 7 days incubation at 25°C. The resultant conidia were suspended in Sabouraud dextrose broth (SDB) and incubated for 2 days at 25°C by shaking at 110 rpm. The resultant liquid culture was mixed with steamed rice at the rate of 10% (v/w) and the mixture was then poured into 15-cm-diameter Petri dishes (100 g per dish). After 7 days growth and conidiation at 25°C, the rice cultures were dried overnight in a ventilation chamber at 33°C and then passed through an electrically vibrating sieve (10 threads mm^{-1}) for harvest of conidia, followed by vacuum drying to ca. 5% water content at ambient temperature (Ye et al. 2006).

The dried conidial powder was uniformly suspended in a mixture of 95% (v/v) industrial paraffin as oil carrier and 5% (v/v) fatty alcohol polyethylene glycol ether 'AEO-3' as emulsifier (Xiaoshan Chemical Additives, Hangzhou, Zhejiang, China). The emulsifiable formulation was standardized to 1×10^{10} conidia ml^{-1} and used immediately or stored at 6°C in dark for bioassays below.

Viability assays at different regimes

Aqueous dilution (1×10^6 conidia ml^{-1}) of the emulsifiable formulation was prepared and 100 µl aliquots were smeared evenly onto the 90-mm-diameter plates of SDAY supplemented with 0.1% chloramphenicol to prevent possible bacterial contamination. Not covered with lids, the smeared SDAY plates were maintained in incubators at 20, 25 and 30 ± 1°C with 12:12 L:D, respectively. Each incubator included three Perspex chambers (135 × 135 × 185 mm), in which 51%, 74% and 95% RH were achieved by pumping continuously moisture-specific air from the last of three rubber-tube-connected jars into each chamber (Feng et al. 1999). Aqueous solutions of 59.8%, 31.4% and 18.8% (v/v) glycerin were separately half-filled into each set of the jars to generate the RHs at 20–30°C (Doberski 1981). The conidia smeared on the plates were thus exposed to nine treatments of temperature and RH combinations, each including three plates as replicates.

Germinated and non-germinated conidia at each of the regimes were counted after 12 and 24 h incubation under microscope at 400× magnification (three counts of >100 conidia per plate). Conidial viability at a given regime was determined as percentages of the germinated conidia (with visible germ tubes) in total.

Preparation of the mite eggs

The eggs of *T. urticae* were prepared using a detached leaf system described by Shi and Feng (2004). A laboratory population of the mite species was maintained on fava bean (*Vicia faba* L.) plants in a walk-in growth room at the regime of 23 ± 2°C and 12:12 L:D. Twenty vigorous adult females arbitrarily taken from the population were transferred to a detached leaf in Petri dish (6.5 cm diameter), in which root hairs grew from the petiole into an agar plate below the leaf. The females were allowed to lay eggs freely for 18 h and then removed. A certain number of eggs (usually 30–40) were left on each leaf to receive treatments as follows. The detached leaf system could support a mite colony for 15 days or so, warranting normal hatch of the mite eggs with no need for leaf change during a bioassay.

Ovicidal assays of *B. bassiana* at different regimes

Aqueous dilutions (1×10^8, 1×10^7 and 1×10^6 conidia ml^{-1}) of the emulsifiable formulation were sprayed onto the eggs on the leaves for inoculation using a non-touch leaf method (Shi and Feng 2004). Briefly, each uncovered dish of the detached leaf bearing the mite eggs was placed on the center of the bottom specimen dish (11 cm diameter) of an Automatic Potter Spray Tower (Burkard Scientific, Uxbridge, Middx, UK) to receive a 2-ml spray of each conidial dilution from its top nozzle at the working pressure of 0.7 kg cm^{-2} (the manufacturer's guide). Separate equal-volume sprays of the three aqueous dilutions resulted in different concentrations of the conidia deposited onto the mite eggs and leaves. Each concentration was determined as no. conidia mm^{-2} using microscopic counts of the conidia deposited onto a glass slip (20 × 20 mm; five 0.2165-mm^2 view fields per slip), which was placed beside the dish under each spray. The same-volume spray of 100-fold aqueous dilution of the liquid carrier alone (i.e., the mixture of 95% paraffin and 5% emulsifier) was included as blank control of the three fungal sprays in each bioassay.

After exposure to the fungal sprays, all the eggs on detached leaves in Petri dishes were covered with lids and maintained overnight at 25°C and 12:12 L:D to favor conidial germination. Subsequently, all the dishes with the sprayed leaves and mite eggs were uncovered and arranged randomly into the regimes of 20–30°C and 51–95% RH (humidity chambers) as described above with each regime including the three fungal concentrations as treatments. Egg hatches were daily examined until no more eggs hatched for three consecutive days at any of the regimes. All non-hatched eggs, together with the detached leaves, were examined under a dissection microscope for verification of fungal infection. Final egg mortalities in different treatments were computed based on the last-day counts of the hatched and non-hatched eggs. All the bioassays were repeated three times during a period of 75 days.

Data analysis

The 12- and 24-h germination rates of the formulated conidia exposed to the temperature and RH regimes were analyzed using two-way ANOVA. Hatched proportions of the mite eggs observed at the concentrations of 0–693 conidia mm^{-2} from the regimes of 20–30°C and 51–95% RH were plotted over post-spray days. Variation in egg mortalities at a given RH or temperature was differentiated among the fungal concentrations by two-way ANOVA. The egg mortalities caused by the fungal sprays at each of the combined regimes were corrected using background mortality in the corresponding blank control and then subjected to probit analysis. A linear concentration- mortality relationship from each analysis was used to estimate median lethal concentration (LC_{50}) and associated 95% confidence limits (CL) as an index for ovicidal activity of the fungal formulation at each regime. An updated version of DPS software (Tang and Feng 2007) was used in all the analyses.

Results

Effects of temperature and RH on the viability of oil-formulated conidia

The viabilities of *B. bassiana* conidia formulated were significantly affected by temperature (12 h: $F_{2,16} = 171.9$, $P < 0.01$; 24 h: $F_{2,16} = 57.0$, $P < 0.01$) but not by RH (12 h: $F_{2,16} = 2.6$, $P = 0.11$; 24 h: $F_{2,16} = 1.9$, $P = 0.18$) among the concerned regimes. The interaction of both

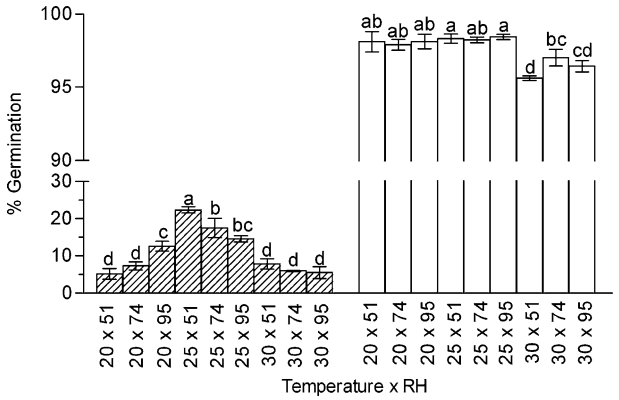

Fig. 1 Comparison of the viabilities of the formulated *Beauveria bassiana* conidia incubated for 12 (shading bars) and 24 h (white bars) on SDAY plates at the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH, respectively. Bars with different letters differed significantly in height (Tukey's HSD, $P < 0.05$). Error bars: SD

variables also had significant effect on the viabilities (12 h: $F_{4,16} = 3.3$, $P = 0.04$; 24 h: $F_{4,16} = 21.7$, $P < 0.01$). Germination ranged from 5.1% at the regime of 20°C and 51% RH to 22.4% at 25°C and 95% RH after 12 h incubation but reached 95.6–98.3% in all the regimes by 24 h (Fig. 1). Thus, the oil-formulated conidia had high viabilities despite some variation perhaps due to the temperature and RH interaction.

After12 h incubation, the overall mean germination rates (± SD) at 51–95% RH were significantly higher at 25°C (18.1 ± 3.6%) than at 20 (8.3 ± 3.5%) or 30°C (6.4 ± 1.5%) (Tukey's HSD, $P < 0.05$). By the end of 24 h incubation, however, mean germination rates in the three RH treatments were very close among the temperatures, i.e., 98.0 ± 0.5% at 20°C, 98.3 ± 0.2% at 25°C, and 96.4 ± 0.7% at 30°C. Moreover, overall mean germination rates at 95% RH (12 h: 10.9 ± 4.3%; 24 h: 97.7 ± 1.0%) did not differ significantly from those at 51% RH (12 h: 11.8 ± 8.1%; 24 h: 97.3 ± 1.4%) or at 74% RH (12 h: 10.2 ± 5.7%; 24 h: 97.7 ± 0.6%) when the three-temperature observations were pooled (Tukey's HSD, $P > 0.05$).

Hatch trends of sprayed mite eggs at different regimes

Sprays of the three conidial dilutions generated mean concentrations of 17.9 (±3.0), 160.4 (±17.1) and 693.1 (±183.6) conidia mm^{-2} deposited on the leaves with 39 (25–76) eggs per capita in the repeated bioassays. Thus, a total number of 4,218 mite eggs sprayed at 0–693 conidia mm^{-2} were exposed to the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH.

The trends of hatched proportions of the mite eggs at all the regimes are illustrated over days after each fungal spray (Fig. 2). Generally, observations within each of the regimes were dependent on both fungal concentrations and post-spray days. Most of the mite eggs in blank controls or sprayed at the low fungal concentration hatched within 7–9 days but no egg hatch was observed in the first 2 days. Differences in hatch rates were small among the fungal treatments during the first 3–4 days but became larger thereafter. Very few eggs were observed hatching from day 9 or 10 onwards. As a result, different numbers of the mite eggs were not hatched in the fungal treatments irrespective of the regimes.

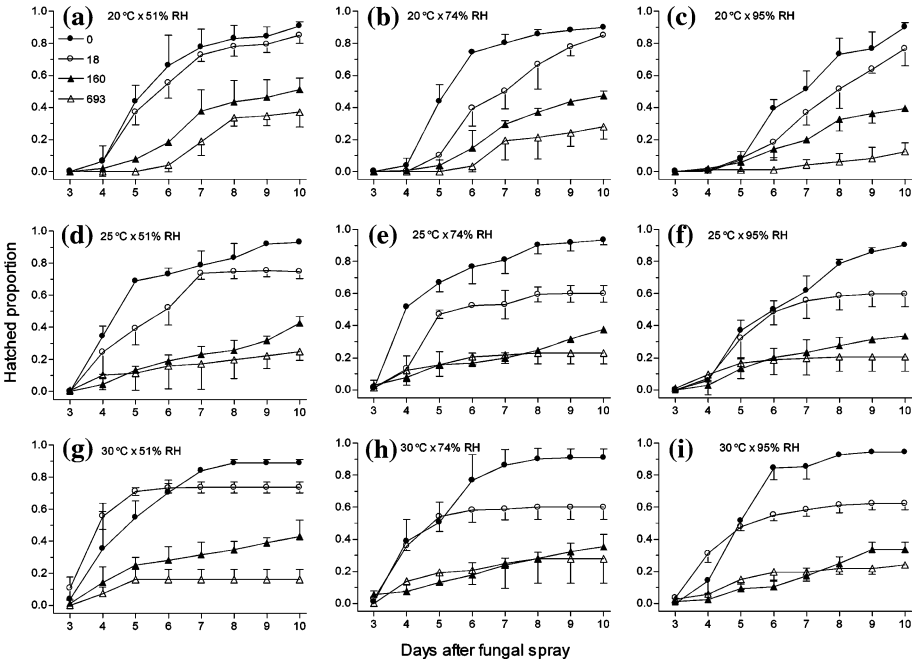

Fig. 2 The trends of the hatched proportions of *Tetranychus urticae* eggs at the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH over days after being sprayed with the emulsifiable *Beauveria bassiana* formulation at the concentrations of 0–693 conidia mm^{-2} (0 = blank control). Error bars: SD

Egg mortalities caused by fungal sprays at different regimes

Variations in the final mortalities of the mite eggs at the different fungal concentrations were differentiated by two-way ANOVA (Table 1). The fungal concentration was consistently most influential on the egg mortalities (maximal F with minimal P) at a given temperature or RH. This indicates that the egg mortalities were attributed to infection by *B. bassiana*. The RH effects on the mortalities were significant only at 20 and 25°C ($P < 0.01$) but insignificant at 30°C ($P = 0.79$). The RH and concentration interaction was not significant at a given temperature ($P > 0.05$). The effect of temperature on the mortalities was significant only at 51% or 74% RH ($P < 0.01$) but not at 95% RH ($P = 0.23$). However, a significant effect was found in the interaction of temperature with the fungal concentration at 95% RH ($P < 0.01$).

The egg mortalities caused by the fungal formulation fell in the range of 62.5–87.9% at the high concentration and of 48.9–66.6% at the medium, varying with the temperature/RH regimes (Fig. 3). These were significantly higher than the background mortalities of 5.6–11.3% in the blank controls (Tukey's HSD, $P < 0.05$). The low fungal concentration resulted in the mortalities of 15.0–40.4% but only those at the regimes of 25°C or 74–95% RH were significantly higher than the mortalities in the controls.

LC_{50}s as indices of ovicidal activities at different regimes

The linear concentration-mortality relationships determined by probit analysis generated the LC_{50} values and associated 95% CL for the tested formulation against the mite eggs at

Table 1 Variation in the mortalities of *Tetranychus urticae* eggs attributed to the sprays of *Beauveria bassiana* formulation (0–693 conidia mm^{-2}) at different temperature and RH regimes

Source of variation	Given temperature			Source of variation	Given RH		
	df	*F*	*P*		df	*F*	*P*
20°C				51% RH			
Replicate	2, 22	0.5	0.60	Replicate	2, 22	0.6	0.56
RH	2, 22	11.9	<0.01	Temperature	2, 22	10.3	<0.01
Fungal spray	3, 22	245.2	<0.01	Fungal spray	3, 22	229.0	<0.01
RH × spray	6, 22	2.3	0.07	Temp × spray	6, 22	1.8	0.15
25°C				74% RH			
Replicate	2, 22	1.0	0.39	Replicate	2, 22	0.5	0.60
RH	2, 22	7.6	<0.01	Temperature	2, 22	7.1	<0.01
Fungal spray	3, 22	335.3	<0.01	Fungal spray	3, 22	166.3	<0.01
RH × spray	6, 22	1.6	0.21	Temp × spray	6, 22	3.3	0.02
30°C				95% RH			
Replicate	2, 22	0.1	0.87	Replicate	2, 22	1.2	0.33
RH	2, 22	0.2	0.79	Temperature	2, 22	1.6	0.23
Fungal spray	3, 22	161.1	<0.01	Fungal spray	3, 22	335.4	<0.01
RH × spray	6, 22	2.3	0.08	Temp × spray	6, 22	4.3	<0.01

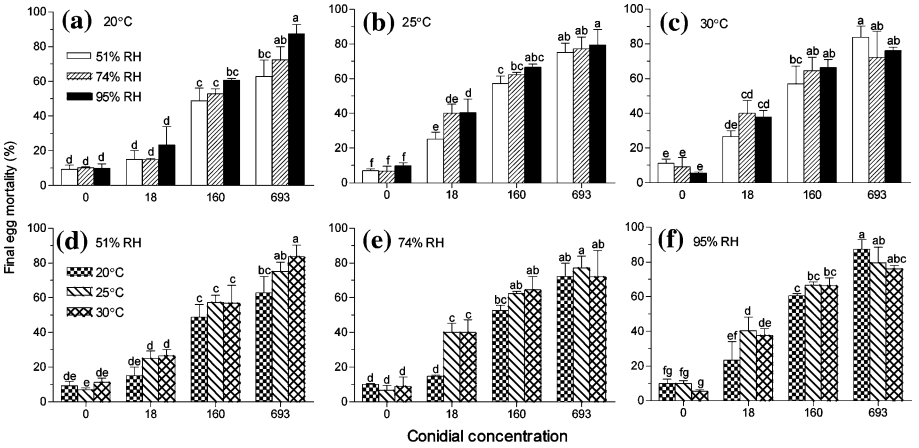

Fig. 3 Comparison of the final mortalities of *Tetranychus urticae* eggs at the different concentrations of *Beauveria bassiana* formulation (no. conidia mm^{-2}; 0 = blank control) at the regimes of 20, 25 and 30°C with 51%, 74% and 95% RH, respectively. Bars with different lowercase letters in each graph differed significantly in height (Tukey's HSD, $P < 0.05$). Error bars: SD

all the regimes (Table 2). The fungal formulation was highly ovicidal with the LC_{50} declining with increased RH at a given temperature. The maximal LC_{50} at the regime of 20°C and 51% RH was 320 conidia mm^{-2}. Those at the regimes of 25–30°C with 74–95% RH were only 65–78 conidia mm^{-2}.

Discussion

In summary, the formulated *B. bassiana* conidia were highly viable at the regimes of 20–30°C with 51–95% RH. The hatched proportions of *T. urticae* eggs after exposure to

Table 2 The LC_{50} estimates and associated 95% confidence limits (CL) for the ovicidal activities of emulsifiable *Beauveria bassiana* formulation against *Tetranychus urticae* at different temperature and RH regimes, based on probit analysis

Temp (°C)	RH (%)	Intercept	Slope ± SE	χ^2	P*	LC_{50} with 95% CL (no. conidia mm^{-2})
20	51	2.35	1.06 ± 0.17	2.40	0.12	319.9 (209.5–593.1)
20	74	2.02	1.26 ± 0.17	2.12	0.15	233.0 (167.9–354.4)
20	95	2.36	1.29 ± 0.15	0.22	0.64	111.0 (80.5–150.9)
25	51	2.99	0.94 ± 0.12	0.10	0.75	135.7 (94.1–197.3)
25	74	3.74	0.68 ± 0.12	0.00	0.99	70.7 (35.6–118.4)
25	95	3.61	0.76 ± 0.11	0.12	0.73	66.1 (37.5–103.4)
30	51	2.47	1.18 ± 0.14	0.29	0.59	136.0 (96.5–193.4)
30	74	3.81	0.63 ± 0.12	0.99	0.32	78.7 (39.6–135.5)
30	95	3.71	0.71 ± 0.11	0.59	0.44	65.4 (36.4–103.2)

* Homogeneity for the fit was accepted if $P > 0.05$ for the χ^2 test (df = 2)

fungal sprays of 18–693 conidia mm^{-2} were generally lower than those in blank controls despite some variations. The final egg mortalities in the fungal treatments were always higher than those in blank controls irrespective of the temperature/RH regimes. The RH effect on the fungal action was significant at 20 and 25°C but not at 30°C whereas the effect of temperature was significant at 51% and 74% RH but not at 95% RH. The ovicidal LC_{50} s of the formulation spanned from 65 to 320 conidia mm^{-2} at all the regimes but fell in a very narrow range of 65–78 conidia mm^{-2} at 25–30°C with 74–95% RH. The results indicate a conspicuous ovicidal activity of the fungal formulation towards the spider mite species at the concerned regimes.

A high viability of fungal conidia in a formulation sprayed onto target pests is a prerequisite for their germination and infection. Germination *in vitro* is related to expression of fungal virulence (Jackson et al. 1989; Altre et al. 1999). Since conidial germination is known to largely depend on RH and temperature (Feng et al. 1994; Roberts and St. Leger 2004) and spider mite eggs are usually laid on the surfaces of leaves or shoots with some moisture (e.g., metabolic water, dew), the formulated conidia in this study were allowed to germinate for 24 h on uncovered SDAY plates entirely exposed to the regimes of 51–95% RH and 20–30°C. These are normal conditions for heavy infestation of spider mite pests in the field. The observed high viabilities help to interpret the high egg mortalities caused by *B. bassiana* at the same regimes. This indicates that the emulsifiable formulation would be able to act on spider mites under field conditions. Other reports have also shown substantial infections of *B. bassiana* and/or *M. anisopliae* to southern pine beetle adults at 55–94% RH (Moore 1973), elm bark beetle larvae at 51–100% RH (Doberski 1981), grasshoppers at 12–100% RH (Marcandier and Khachatourians 1987), and the Chagas' disease vector *Rhodnius prolixus* Stål at 43–97% RH (Fargues and Luz 2000), despite higher mortalities associated with higher RH.

The LC_{50} of the tested *B. bassiana* formulation against *T. urticae* eggs ranged from 65 conidia mm^{-2} at 30°C and 95% RH to 320 at 20°C and 51% RH. We think that the emulsifiable formulation has greatly enhanced ovicidal activities of the fungal agent in comparison with an LC_{50} of 548 (393–857) or 546 (406–818) conidia mm^{-2} (plain conidia suspended in 0.02% Tween-80) toward the eggs of the same mite species at 25°C under moist conditions (Shi and Feng 2004; Shi et al. 2005). This supports previous reports on oil-increased efficacy of *M. anisopliae* against whiteflies (Malsam et al. 2002) and of *B. bassiana* against aphids (Ye et al. 2005), and on improved adaptation of oil formulations to low-humidity environ-

ments for insect control (Bateman et al. 1993; Kooyman and Godonou 1997). Although possible mechanisms involved in the enhancement of fungal activities by the oil-based formulation are not clear at present, we postulate that the enhancement may result from better attachment of the formulated conidia to target pests and from improved protection of the conidia from desiccation after spray. This warrants more studies.

The ovicidal activities of the emulsifiable formulation tested at different temperature/RH regimes highlight its potential for practical incorporation into mite pest management due to its adaptation to the low-RH environments. In field trials, this formulation sprayed twice at the rate of ca. 1.5×10^{13} conidia ha^{-1} has provided significant control of citrus red mites, *Panonychus citri* (McGregor), in the orchards of east China (Shi and Feng 2006) and of cotton spider mites, mainly *Tetranychus truncates* Ehara and *T. turkestani* (Ugarov & Nikolskii), in the Tarim Basin of northwest China (Shi et al. 2008b). However, spider mites in southern China and other subtropical areas often infest crops heavily during hot summer, which is a challenge for the tolerance of the fungal formulation to outdoor thermal stress often around 40°C. If fungal candidates with greater thermotolerance and other improved traits (Ying and Feng 2004; Zou et al. 2006) are formulated into the oil-based carrier, application of fungal formulations to more stressed seasons or environments for spider mite control would be more promising. This also warrants future studies.

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