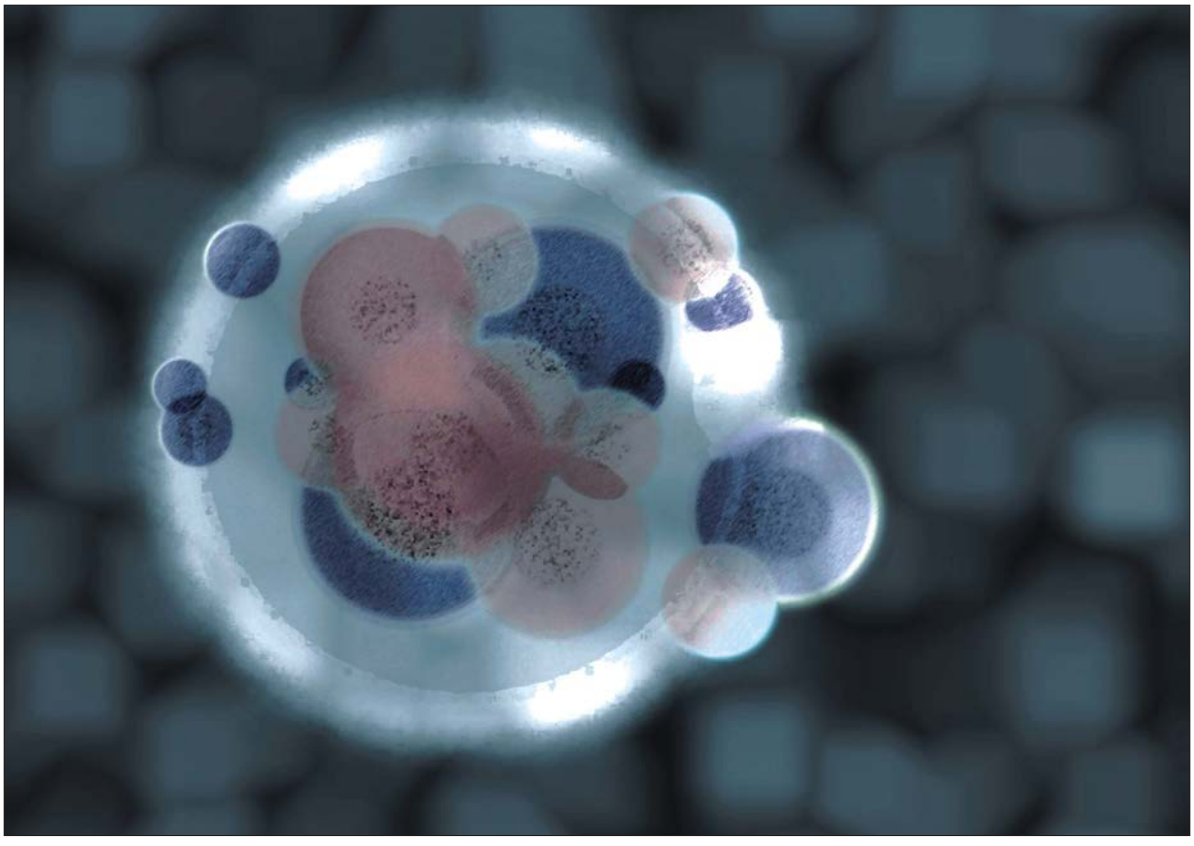

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Variation in Germination, Virulence and Conidial Production of Single Spore Isolates of Entomopathogenic Fungi in Response to Environmental Heterogeneity

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Abstract: The objectives of this study were to evaluate spore germination, virulence against a surrogate host [the western flower thrips, *Frankliniella occidentalis* (Pergande)] and spore production capabilities at different temperatures and RH combinations of 6 single spore isolates newly obtained from multispore isolates of *Beauveria bassiana* (Balsamo) Vuillemin (5672) and *Metarhizium anisopliae* (Metschnikoff) (1080). We found that temperature and RH influenced conidial germination, spore production and virulence of 5672 and 1080 and their single spore isolates tested. At =98% RH, 20 and 30°C, single spore isolates from 5672 had higher %germination than their multispore isolate, while at 100% RH and all temperature tested, the single spore isolates (1080-1 and 1080-2) had higher germination than their multispore isolate 1080. At 95% RH, the conidial germination was poor and only occurred at 20°C for two single spore isolates (5672-4 and 5672-5) as well as for the multispore isolates 5672 and 1080. *Metarhizium anisopliae* isolates were more virulent against western flower thrips than the *B. bassiana* isolates tested. At 100% RH, mortality of western flower thrips was more obvious than at other %RH levels tested. Single spore isolates 5672-1 and 5672-3 were less virulent than other isolates tested, whereas 1080-3 and 1080-1 were more virulent than all tested isolates. Conidial production at 100% RH and 20°C was higher than other %RH and temperatures tested. At 95% RH and temperatures of 30 and 15°C, conidial production was limited to few isolates. Only 5672-5 produced conidia at 30°C, and 5672-1, 5672-4, 5672-5 and the multispore isolate 5672 produced conidia at 15°C, but no production of conidia was found for the multispore 1080 and its single spore isolates at these temperature and RH combinations. No conidial production was found at <95% RH. Variability in conidial germination, spore production and virulence of 5672 and 1080 and their single spore isolates tested will help in selection and development of these fungi for Sunn Pest, *Eurygaster integriceps* Puton management under temperatures and RH levels occurring in the Sunn Pest overwintering sites.

Key words: *Beauveria bassiana*, *Metarhizium anisopliae*, temperature, relative humidity, single spore isolates, germination, spore production, virulence

INTRODUCTION

Sunn Pest is a term referring to a group of three dozen or more species belonging to a number of genera of the families Scutelleridae and Pentatomidae in the suborder Heteroptera. They cause considerable damage to cereal crops throughout the Middle and Near East and most of the former USSR (Critchley, 1998). The key species of the Sunn Pest group is *Eurygaster integriceps* Puton, an important insect pest of wheat and barley (Banks *et al*., 1961; Critchley, 1998). Wheat yield losses caused by this pest are estimated to be 20-70% and damage can reach 100% if control methods are not applied (Skaf, 1996). If 2-3% of the grains are damaged by Sunn Pest, the grains are not useful for baking (El-Haramein *et al*., 1984). Sunn Pest has one generation per year, spending 2.5-3 mo on graminaceous plants and around 9 mo under the litter of pine or oak trees in overwintering sites. These sites are located in the hills and mountains close to wheat and barley fields (Banks *et al*., 1961). Trials in greenhouses and overwintering sites using selected isolates of entomopathogenic fungi showed promising results based on the mortality of Sunn Pest adults. These assays indicated that mortality of Sunn Pest ranged from 66.1->95% and 50-91% in litter and on plants, respectively (Parker *et al*., 2003).

Chemical control, using aerial applications, is commonly used for Sunn Pest management throughout its range. This has adverse effects on beneficial insects and non-target organisms. Entomopathogenic fungi have achieved different levels of success as biological control agents against greenhouse, field, pasture, tree fruit, forest and urban pests (Inglis *et al*., 2001).

A successful microbial insecticide should be capable of a high spore germination rate and possess a high spore production ability, moreover, it should be virulent against the target insect pest (Daoust and Roberts, 1983; Goettel and Roberts, 1992; Soper and Ward, 1981). Temperature and relative humidity (RH) affect germination and spore production of entomopathogenic fungi (Arthurs and Thomas, 2001; Carruthers and Hural, 1990; Sivasankaran *et al*., 1998). These environmental factors also affect the virulence capability of entomopathogenic fungi (Vidal *et al*., 2003).

This research is part of a program for the development of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) for Sunn Pest management. We evaluated spore germination, virulence against *Frankliniella occidentalis* (Pergande) (western flower thrips) and spore production capabilities at temperatures and RH levels that occur in the Sunn Pest overwintering sites. Because Sunn Pest is not available in North America, the western flower thrips was used in the virulence test. We did this research using 6 single spore isolates newly obtained from each *B. bassiana* (5672) and *M. anisopliae* (1080). The single spore isolates were used to investigate the variability of these fungi in response to environmental conditions. Characterizing this variability facilitates selection of fungi with desired characteristics, such as high spore germination, production and virulence, under relevant temperatures and RH levels. This will help in the development of entomopathogenic fungi by overcoming the challenges associated with using fungi in overwintering sites for management of Sunn Pest.

MATERIALS AND METHODS

Germination

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, *Heliocoverpa 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 ¼ 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 distilled H_2O and water 1L and incubated at 24±2°C for 10 days.

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 with small glass balls (1-2 mm diam.). The suspension was vortexed for approximately 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). Series of dilutions were made from each suspension until the final suspension contained an estimated 10 conidia mL^{-1}. From the final suspension of each isolate, 1 mL was pipetted onto the surface of a 9-diam Petri dish with SDAY/4 medium and held at 25°C.

The procedure for selection of single spore isolates was as follows. After 24 h, conidial germination was inspected under a microscope at 100×magnification and individual germinated conidium was coded and marked by drawing a circle around each on the bottom of the petri dish. This procedure was done to ensure that each developing colony was initiated from a single conidium. After 3 days a tiny piece of the fungal growth from one single marked colony was transferred onto the surface of 9-cm diam Petri dish containing SDAY/4 medium. This procedure was repeated for 6 single spore isolates from each 5672 and 1080 isolates. Petri dishes were held at 25°C in the dark. After 2 weeks, approximately 10 mL of 0.01% Tween 80 was added to each single spore isolate culture and mixed thoroughly until a homogenous suspension was achieved and 0.1 mL of each suspension was transferred into a petri dish containing SDAY/4 and incubated at 24±2°C for 1 week. These fresh cultures were used for further experiments. Single spore isolate cultures were held at 4°C as a permanent source for further tests. For *B. bassiana* isolate 5672, single spore isolates were labeled as 5672-1, 5672-2, 5672-3, 5672-4, 5672-5 and 5672-6. For *M. anisopliae* isolate 1080, single spore isolates were labeled as 1080-1, 1080-2, 1080-3, 1080-4, 1080-5 and 1080-6.

Germination assessment: A stock suspension of 1×10^6 conidia mL^{-1} from each of the mixed spore isolates of 5672 and 1080 and their single spore cultures was prepared as described above. 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 treatment combinations. In this test,

6-cm-diam petri dishes were used. In each petri dish, 5 drops (40 μL) of SDAY/4 media were used as a germination test substrate. 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}) was pipetted on each drop. Petri dishes were allowed to dry under the sterile hood for an additional 30 min before temperature and RH exposure.

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 and Thomas, 2001). 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. Metal frames attached with twist ties held experimental Petri dishes in place on the platform. 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). 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 h post exposure. Germination was stopped by adding one drop of lacto phenol cotton blue on each of the 5 drops of substrate in a petri dish. The substrate drops 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 dish (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 percentage of germination. The experiment was repeated three times.

Virulence assay against western flower thrips: Petri dishes (35×10) mm were used in this experiment. To obtain the same %RH inside each petri dish, a 2 mm diam hole was made in the center of the lid. To prevent the bioassay organisms (western flower thrips) from escaping, the hole was covered with a 3 mm diam disc of 118-micron nylon screening affixed using methyl ethanol as a gluing agent. A disc of a 10 days old bean leaf, *Phaseolus vulgaris* L. var. Royal Burgundy (Agway, Inc., Box 4933, Syracuse, NY 13221) (35 mm diam) was placed inside each petri dish. Three milliliters of the 1×10^6 conidia mL suspension from each single spore isolate culture and from each of the mixed culture of both fungi, as well as from a suspension of 0.01% Tween 80 (control), were individually showered onto the upper surface of the leaf disc using a Potter spray tower (Burkard Ltd., Hertfordshire, UK). Petri dishes were left for 20 min to dry. Ten-second instar western flower thrips were then placed on the leaf surface. The petri dish was covered with the vented lid and sealed with Parafilm M®. Petri dishes were held at the required temperature and %RH combinations as described above. Mortality assessment was made 1 week after treatment. Western flower thrips was mass reared at the Entomology Research Laboratory at the University of Vermont (Burlington). They were produced in a laboratory colony set up for even age production (Doane *et al*., 1998).

Spore production: From each isolate, 10 μL of 1×10^6 conidia mL suspension was added to the center of a 6–cm diam Petri dish containing 100 μL SDAY/4 media dried for 50 min under sterile hood conditions. Petri dishes were held at 15, 20 and 30°C and 91, 95, 98 and 100% RH combinations as described above. After 20 d, 10 mL 0.01% Tween 80 were added and a growing colony was harvested with spatula and placed into 10 mL 0.6% Green shield and 0.1% Tween 80. This was sonicated for 10 min to separate conidia from mycelia (Parker *et al*., 2003). Spore concentrations were determined by taking the average of two readings using a Levy hemocytometer (Hausser Scientific, Horsham, PA USA) 0.100 mm deep.

Data analysis: SAS PROC GLM (SAS Institute, 2002) was used for data analysis. The experimental design was factorial with three factor levels (temperature, RH and isolates). Three runs were made for each experiment with one replication per run. Up to three way interactions were evaluated at $\alpha = 0.05$.

RESULTS

Spore germination: Conidial germination of the multispore isolates of *B. bassiana* (5672) and *M. anisopliae* (1080) as well as 6 single spore isolates obtained from each of them was influenced by isolate, temperature and %RH tested. However, a significant three-way interaction was found between these parameters ($p = 0.05$) (Table 1).

Table 1: Analysis of variance for conidial germination of multispore isolates of *B. bassiana* (5672) and *M. anisopliae* (1080), as well as 6 single spore isolates obtained from each of them, at RH of 98 and 100±1% and temperatures of 15, 20, and 30±1°C. Data from RH 91 and 95% not included in analysis

Source	df	Type III SS	Mean square	F-value	p >F
Run	2	8.22222	4.11111	2.94	0.0558
RH	1	71609.14	71609.14	51139.6	<.0001
Temperature	2	83436.44	41718.22	29793	<.0001
[1]Isolate	13	10800.49	830.8071	593.32	<.0001
RH* Isolate	13	7681.19	590.8608	421.96	<.0001
RH* Temperature	2	43779.31	21889.65	15632.5	<.0001
Temperature * Isolate	26	12973.01	498.9618	356.33	<.0001
RH*Temperature* Isolate	26	27369.69	1052.68	751.77	<.0001
Error	166	232.4444	1.4003		
Total	251	257889.9			

[1]Represents both multispore isolate and 6 single spore isolates from each

Table 2: Analysis of variance for the vimlence of multispore isolates of *B. bassiana* (5672) and *M. anisopliae* (1080) as well as 6 single spore isolates obtained from each, at RH of 98 and 100±1% and temperatures of 15, 20, and 30±1°C

Source	df	Type III SS	Mean square	F-value	p >F
Run	2	2485.378	1242.689	6.26	0.0021
%RH	3	6798.778	2266.259	11.41	<.0001
Temperature	2	569124.9	284562.4	1433.09	<.0001
Isolates	14	50550.07	3610.719	18.18	<.0001
Temperature* Isolates	28	46155.54	1648.412	8.3	<.0001
%RH* Isolates	42	13411.32	319.3173	1.61	0.0123
%RH*Temperature	6	3651.103	608.5172	3.06	0.0062
%RH*Temperature* Isolates	84	24447.49	291.0415	1.47	0.0094
Error	358	71086.53	198.5657		
Total	539	787711.1			

[1]Represents both multispore isolate and 6 single spore isolates from each

Table 3: Analysis of variance for conidia production of *B. bassiana* (5672) and *M. anisopliae* (1080) as well as 6 single spore isolates obtained from each of them at RH of 98 and 100±1% and temperatures of 15, 20, and 30±1°C. Data from 91 and 95% RH were not included in the analysis

Source	df	Type III SS	Mean square	F-value	p >F
Run	2	41167924062	20583962031	1.87	0.1569
%RH	2	4941799400000	2470899700000	223.97	<.0001
Temperature	2	4064130400000	2032065200000	184.19	<.0001
[1]Isolates	13	887739320632	68287640049	6.19	<.0001
%RH* Isolates	26	1108874300000	42649009626	3.87	<.0001
%RH*Temperature	4	3069849800000	767462445583	69.56	<.0001
Temperature* Isolates	26	1156251900000	44471228068	4.03	<.0001
%RH*Temperature* Isolates	52	1820582200000	35011196841	3.17	<.0001
Error	250	2758080100000	11032320251		
Total	377	19848475000000			

[1]Represents both multispore isolate and 6 single spore isolates from each

In all cases, no fungal germination occurred at RH<95% at all temperatures tested, while little germination was observed at 95% RH and 20°C for 5672, 5672-4, 1080, 1080-5 isolates with germination rate 1, 1.3, 0.6 and 1%, respectively (Fig. 1A and B). For this reason, germination data from ≤95% RH were not included with data analysis.

At 98% RH, low or intermediate germination rates were observed among single spore isolates and their mutispore isolates tested. For 5672 isolates, the germination rates ranged from 2-31, 9-51 and 9-52% at 30, 20 and 15°C, respectively. For 1080 isolates, conidial germination ranged from 1-22%, 1-32% and 1-5% at 30, 20, 15°C, respectively. Fluctuating trends in conidial germination at this RH occurred depending on temperate and isolate interaction.

At 100% RH, conidial germination was high in general. For 5672 isolates, the germination rates ranged from 73-97.6%, 28-72% and 4-12% at temperatures of 30, 20 and 15°C, respectively (Fig. 1A). Whereas for 1080 isolates, the germination rates ranged from 36-90, 24-96 and 0.6-1% at 30, 20 and 15°C, respectively (Fig. 1B). At 15°C, more conidial germination occurred with 5672 isolates than 1080 isolates. All 5672 isolates preformed similarly at 30°C but germination varied with the 1080 isolates. Obvious fluctuating trends occurred for all fungi tested at 20°C (Fig. 1A and B).

Virulence assay against western flower thrips: Higher order interactions occurred in the assessment of virulence capability of single spore isolates and their multispore isolates as influenced by temperature and %RH treatments (Table 2). At 30°C, the mortality of western flower thrips caused by 5672 isolates was high and ranged from 55-100, 60-100, 67-100 and 68.7-100% at 91, 95, 98 and 100±1% RH, respectively (Fig. 2 A). At 20°C, the mortality ranged from 12.5-28.5, 0-29, 0-37.5 and 21-37.5% at 91, 95,

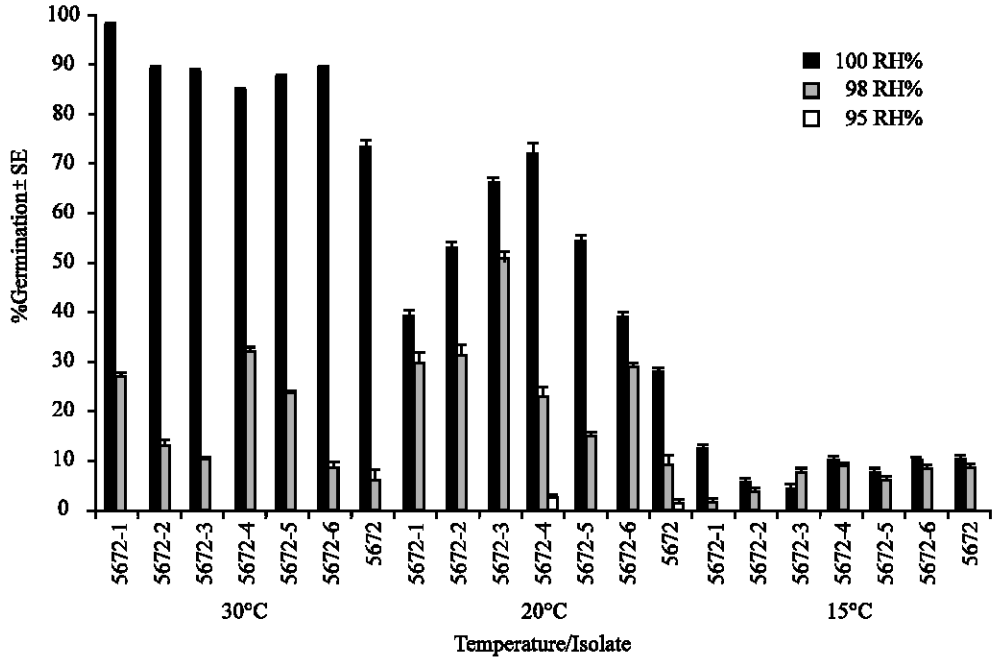

Fig. 1A: Germination of multispore isolate of *B. bassiana* (5672) and 6 single spore isolates obtained from it, at RH 95, 98 and 100±1% and temperatures of 15, 20 and 30±1°C

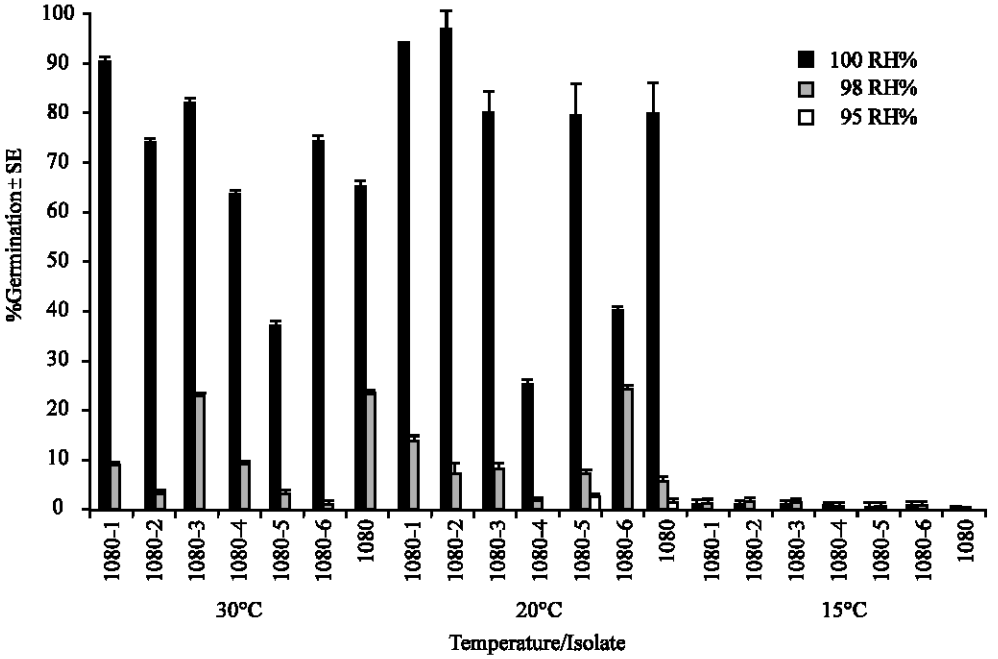

Fig. 1B: Germination of multispore isolate of *M. anisopliae* (1080) and 6 single spore isolates obtained from it, at RH of 95, 98 and 100±1% and temperatures of 15, 20 and 30±1°C

98 and 100±1% RH, respectively (Fig. 2A). At 15°C, the mortality ranged from 0-14, 0-16.6, 0-16.6 and 7-24.2% at 91, 95, 98 and 100±1% RH, respectively (Fig. 2A). The mortality rates of western flower thrips by 1080 had similar trends as 5672 and fluctuated depending on temperatures and %RH regimes tested. At 30°C the mortality ranged from 50-100, 70-100, 75-100 and 100% at 91, 95, 98 and 100±1% RH, respectively (Fig. 2B), while at

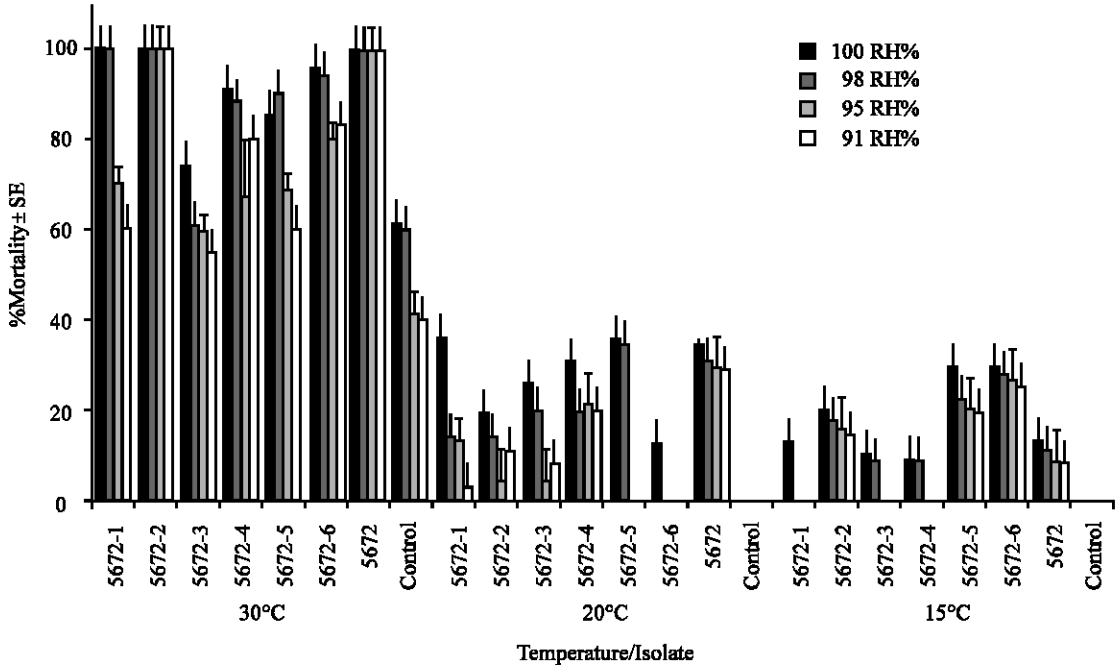

Fig. 2A: Percent of mortality of western flower thrips by multispore isolate *B. bassiana* (5672) and 6 single spore isolates obtained from it at temperatures of 15, 20 and 30±1°C and RH of 91, 95, 98 and 100±1% RH after 7 d of exposure. Data for controls are shared for *B. bassiana* and *M. anisopliae*, which were run concurrently

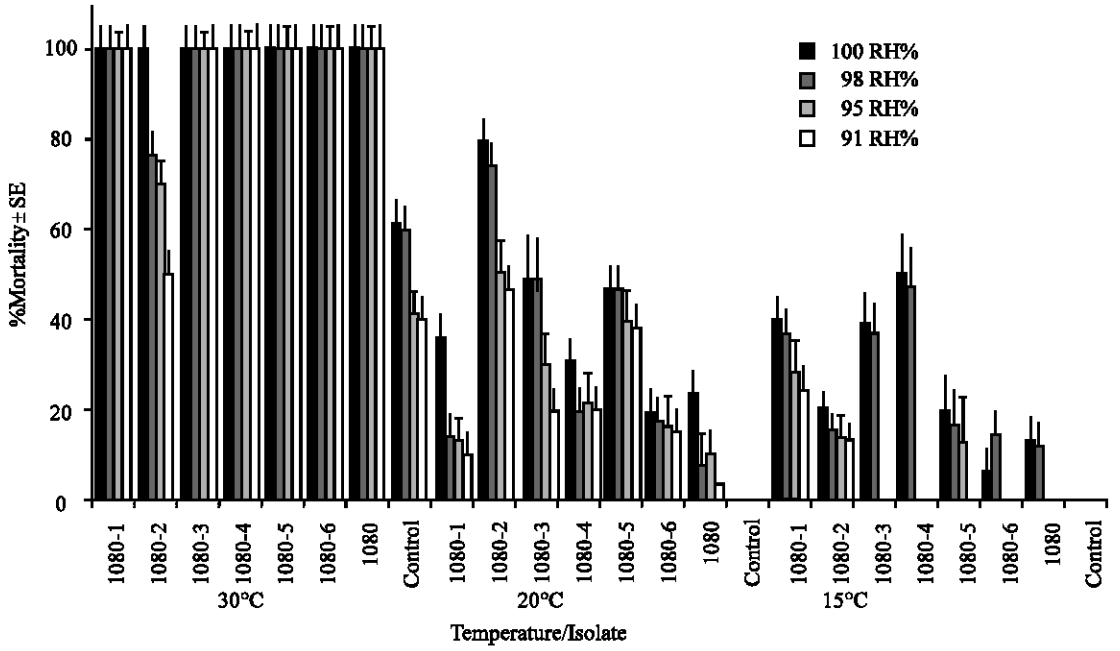

Fig. 2B: Percent of mortality of western flower thrips by multispore isolate *M. anisopliae* (1080) and 6 single spore isolates obtained from it at temperatures of 15, 20 and 30±1°C and RH of 91, 95, 98 and 100±1% RH after 7 d of exposure. Data for controls are shared for *B. bassiana* and *M. anisopliae*, which were run concurrently

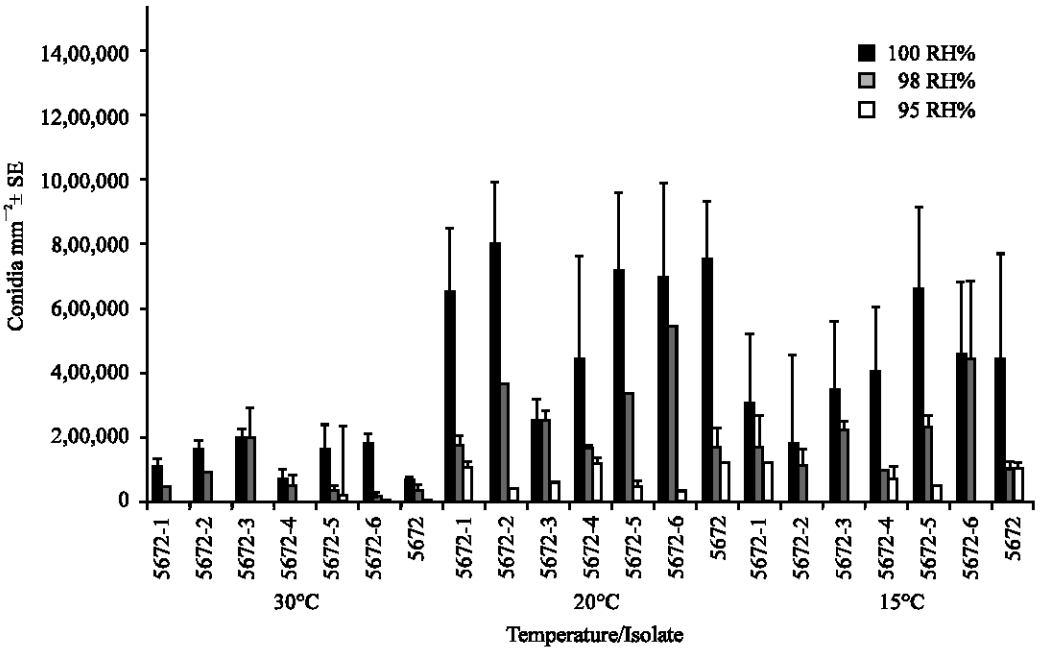

Fig. 3A: Spore production of *B. bassiana* (5672) and 6 single spore isolates derived from it, under RH (95, 98 and 100±1%) and temperatures (15, 20 and 30±1°C)

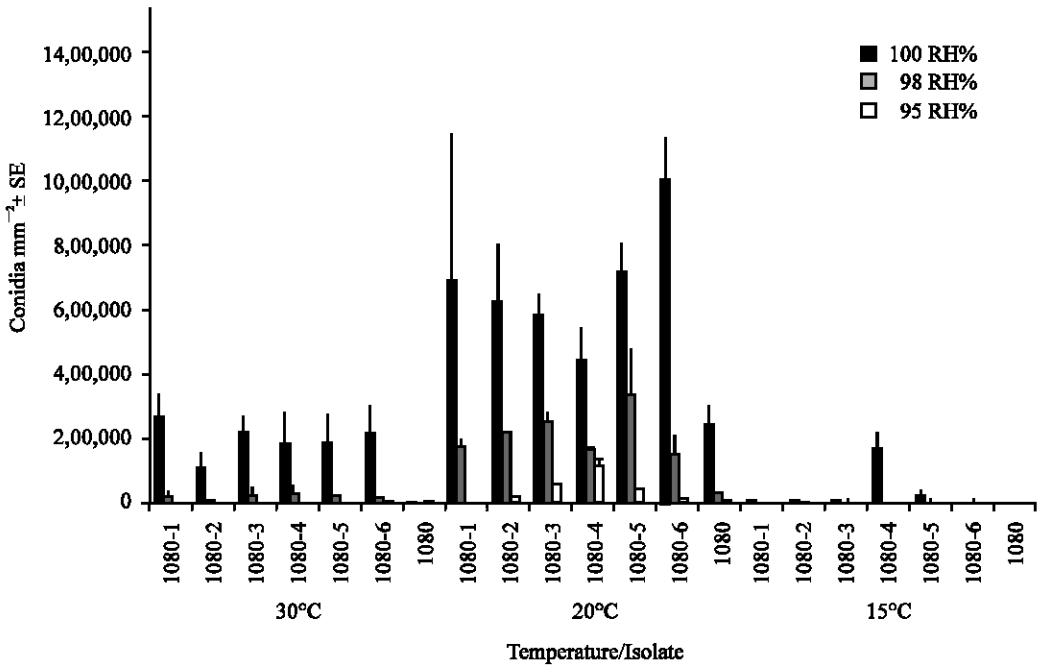

Fig. 3B: Spore production of *M. anisopliae* (1080) and 6 single spore isolates derived from it under RH (95, 98 and 100±1%) and temperatures (15, 20 and 30±1°C)

20°C it ranged from 2-45, 2-50, 5-75 and 25-80% at 91, 95, 98 and 100±1%RH, respectively (Fig. 2B). At 15°C the mortality rates were low and ranged from 0-20, 0-28.5, 8.3-37.5 and 6.35-38.5% at 91, 95, 98 and 100±1%RH, respectively (Fig. 2B). Data for controls are shared for 5672 and 1080, which were run concurrently. Mortality of western flower thrips in the control treatment was less than the other treatments, although at 30°C it was high 38, 39, 59 and 59.5% at 91, 95, 98 and 100% RH, respectively. At 30°C, for the 1080 isolates tested, %RH was not a vital factor in the mortality of insects because high mortally rates occurred at all %RH levels tested. This might be related to high temperature effects on western flower thrips or to long exposure period. Variation in insect mortally was observed at 20 and 15°C for all isolates tested (Fig. 2A and B). The trends in this test did not match well with that in the germination test, except that, at 20°C, 1080-2 tended to perform better than other isolates. Regardless, the fluctuations still exist among single spore isolates in both tests.

Spore production capabilities: We found that temperature and %RH influenced the conidial production of the single spore isolates from the isolates *B. bassiana* (5672) and *M. anisopliae* (1080) (Table 3). Significant higher order interactions ($p<0.05$) occurred among temperature, %RH and isolate factors (Table 3). No conidial production occurred for any isolates of either entomopathogenic fungi at RH<95%. No conidial production for the 1080 isolates occurred at 95% RH and 15 or 30°C, while only few single spore isolates of 1080 produced at 20°C (Fig. 3B). Therefore, data from 91 and 95% RH were excluded from data analysis for 5672 and 1080 isolates tested.

In general, high %RH was needed for spore production of all isolates tested, especially for *M. anisopliae* (1080) and its single spore isolates (Fig. 3B). However, all isolates tested produced more conidia at 100% RH than 98 and 95% RH at all temperatures tested (Fig. 3A and B). Conidial production for fungal isolates tended to fluctuate at temperatures and %RH tested. However, conidial production of the multispore isolate (1080) was less than for its single spore isolates tested at all experimental parameters (Fig. 3B). At all %RH levels tested, conidial production of 5672 and 1080 isolates tended to be more at 20°C than at other temperatures tested (Fig. 3A and B). While low or no spore productions occurred for 1080 isolates at 15°C (Fig. 3B). These results concur with results from the germination test.

DISCUSSION

Germination: Germination of entomopathogenic fungi is dependent on temperature and RH (Arthurs and Thomas, 2001). We found that temperature and RH required for high conidial germination differs depending on the isolates tested of the same entomopathgenous species. Significant three way interactions ($p<0.05$) were found between temperature, %RH and isolates. This is evident in the fluctuating trends in conidial germination for single spore isolates of *B. bassiana* (5672) and *M. anisopliae* (1080) tested (Fig. 1A and B).

The highest germination of conidia occurred at 100% RH and temperatures of 30 and 20°C whereas, the conidial germination was dramatically lower at 95% RH and 15°C especially with 1080 isolates (Fig. 1A and B). Previous research found that, at 100% RH, the conidial germination of *B. bassiana* on *Rhodnius prolixus* Stål occurred at temperatures between 15-35°C and the highest germination occurred at temperatures ranging from 25-30°C (Luz and Fargues, 1998). In another study, the highest conidial germination (99.1-100%) for several isolates of *B. bassiana* occurred at temperatures of 20-30°C but no germination was found at 15° and 35°C (Junianto and Sri, 1995). In the same study, at RH between 92.5-100%, the germination was high for all isolates tested, where germination did not occur at a RH of 75-85%. Huafeng *et al*., (1998) found that at 25°C and 95% RH, conidia of *B. bassiana* germinated on *Dendrolimus punctatus* Walker with high germination and penetration rates. McCammon and Rath (1994) found that the conidial germination for 122 isolates from 16 *M. anisopliae* subsp. *anisopliae* strains was different over a range of temperatures (2.5-37°C). They obtained strains of *M. anisopliae* that are able to germinate and cause infection at 5°C.

In our study, we found variability in conidial germination not only among isolates but also within the single spore isolates obtained from the same isolate. For example, at 100% RH, the conidial germination of the single spore isolate (5672-4) was generally higher than for other single spore and multispore isolates tested at temperatures of 20 and 15°C, whereas at 30°C, conidia of single spore isolates (5672-1) had higher germination than related single spore and multispore isolates tested (Fig. 1A). At 98% RH germination of single spore isolate 5672-4 was higher than for other single spore and multispore 5672 isolates at 15 and 30°C; whereas, at 20°C, conidial germination of the single spore isolate 5672-3 was higher than for other single spore and multispore isolates of 5672 (Fig. 1A).

At 95% RH, the conidial germination was poor and only occurred at 20°C for two single spore isolates (5672-4 and 5672-5) as well as for the multispore isolates 5672 and 1080. However, conidial germination of the 5672-4 was higher than other germinated isolates at 95% RH (Fig. 1A and B). There was no conidial germination at <95% RH across all temperatures examined and for all single spore isolates tested. This result is in agreement with Hallsworth and Magan (1999) when they investigated the effects of humidity and temperature in relation to growth of the entomogenous fungi *B. bassiana*, *M. anisopliae* and *P. farinosus*. In their study, they found that the spore germination, germ tube extension and infection for most entomopathogenic fungi require at least 95% RH at the insect surface (Hallsworth and Magan, 1999).

At all %RH tested and at 15°C, *B. bassiana* had a tendency to germinate more than *M. anisopliae,* but this trend was not observed at other temperatures tested (Fig. 1A and B). We concluded that low temperatures were not preferred for conidial germination of *M. anisopliae* isolates tested. This information can be used for timing application in field conditions.

Virulence assay against western flower thrips: We found that temperature and %RH affect the virulence of *B. bassiana* and *M.* amisopliae. A significant three way interaction ($p = 0.05$) occurred between isolate, %RH and temperature tested on the virulence of 5672 and 1080 isolates. Mortality at 100% RH generally was higher than mortality at other %RH levels tested. However, within a specific temperature and isolate combination, there were few differences in mortality of western flower thrips < 98% RH. Mortality at 30°C was higher than other temperatures tested followed by 20°C, although control mortality was also increased. Hastuti *et al.* (1999) investigated the effects of temperatures (15-30°C) and %RH (100, 92, 85, 75.5 and 53%) on the mortality of *Paropsis charybdis* Stål by *B. bassiana.* They found that the mortality at 100% RH was significantly higher than 92, 85, 75.5 and 53%. However, *B. bassiana* caused mortality at most temperatures tested. We found that at all temperatures and RH combinations tested 5672-1 and 5672-3 were usually less virulent against western flower thrips than other 5672 single spore isolates and the original multispore 5672 isolate. Another study found that the virulence of 28 single-spore isolates of *B. bassiana* to larvae of *Galleria mellonella* (L.) was higher than the multispore isolate in laboratory tests (Samsináková and Kálalová, 1983). It is important to make several single spore isolates from an original multispore culture and evaluate their virulence against the target insect and then select the best single spore isolate (Jenkins *et al.*, 1998). In general, we found that *M. anisopliae* isolates were more virulent than those from *B. bassiana* (Fig. 2A and B). Sharma *et al.*, (1999) found the same results when they investigated the virulence levels of the entomopathogenic fungi *B. bassiana, B. brongniartii* (Saccardo) and *M. anisopliae* against *Maladera insanabilis* Brenske and *Holotrichia consanguinea* Blanchard. *Beauveria bassiana* showed weaker virulence against these insects than *B. brongniartii* and *M. anisopliae*.

In conclusion, selection of single spore isolates for field deployment would depend on when it would be used. For instance, 1080-2 had reduced virulence at higher temperatures and lower humidity so it might be less favorable during warmer periods. *Beauveria bassiana*, isolate 5672-2 might be suited to warmer months as it is highly active at all %RH tested. However, these results should be verified against Sunn Pest under overwintering conditions.

Spore production capabilities: We found variability in the conidial production capability of the *B. bassiana* (5672) and *M. anisopliae* (1080) isolates and their derived single spore isolates at specific temperature and %RH combinations tested. This variability is an important factor that should be considered in mass production for large-scale application purposes. We found that conidial production was dependent on temperature, %RH and isolates. For instance, for all isolates tested, spore production at 100% RH and 20°C was higher than at other %RH and temperatures tested. At 95% RH, only 5672-5 produced conidia at 30°C and 5672-1, 5672-4, 5672-5 and the multispore isolate (5672) produced conidia at 15°C. No production of conidia was found for the multispore *M. anisopliae* and its single spore isolates at 95% RH and temperatures of 15 and 30°C (Fig. 3).

Most previous studies investigated spore production of *B. bassiana* and *M. anisopliae* under either different temperature or %RH levels; however, few investigated spore production of these fungi under both these environmental factors. Moreover, most of these studies used insect cadavers as spore production substrates. The dearth of published results may result from proprietary research done for commercial purposes. In our study, we found that *B. bassiana* (5672) and *M. anisopliae* (1080) as well as 6 single spore isolates derived from each of them did not sporulate and produce conidia on dried SDAY/4

medium at <95% RH. We used a dried SDAY/4 medium as a spore production substrate to insure that there was no additional humidity involved in spore production other than the target %RH that we generated using saturated salt solution. Arthurs and Thomas (2001) found that conidial production of *M. anisopliae* var. *acridum* on mycosed cadavers of *Schistocerca gregaria* (Forskal), during 10 day incubation, was optimial at RH = 96% and at temperatures between 20 and 30°C. *Beauveria bassiana* produced conidia when exposed to RH from 75% to 100% on *Diatraea saccharalis* F. larvae (Lepidoptera: Pyralidae), but conidiogenesis did not occur on *Nezara viridula* L. and *Piezodorus guildini* (Westwood) (Sosa-Gómez and Alves, 2000). Sivasankaran *et al.* (1998) found that biomass production and sporulation of *B. bassiana* were greater at 100% RH. Walstad *et al.*, (1970) reported high sporulation of *B. bassiana* and *M. anisopliae* at 100% RH and 25-30°C.

In conclusion, we found that temperature, %RH and isolate interacted in their influence on germination, virulence and conidial production of entomopathogenic fungi. That is to say, these two important environmental factors depended on the isolates tested. Although a fluctuation of conidial reaction to temperature and %RH was found in all tests, the trends in the germination test matched that of spore production but not the results in the virulence test. To be precise, at all %RH tested, *B. bassiana* (5672) and its single spore isolates tested were likely to germinate or produce more conidia at 15°C than for *M. anisopliae* (1080) and its single spore isolates. Moreover, at 95% RH, few isolates of both fungi germinated or produced conidia and only at 20°C, the most favorable temperature. No germination or conidial production happened <95% RH. There was a tendency in the virulence test for mortality of western flower thrips to occur at 91 and 95% RH whereas little or no fungal growth occurred in the germination and spore production tests. This may be related to micro-environmental effects associated with moisture released directly from the excised bean leaf or live insects that slightly raised the %RH in the immediate area of the conidia, for which the saturated salt solutions could not instantly compensate.

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