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Resilience and relative virulence of strains of entomopathogenic fungi under interactions of abiotic stress

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The objective of this study was to examine the effect of interacting conditions of water stress (0.995-0.96 water activity; a_w), elevated temperature (25-37°C) and CO_2 (350, 1000 ppm) on growth and sporulation of strains of three entomogenous fungi, *Beauveria bassiana, Metarhizium anisopliae* and *Isaria farinosa*. Subsequently, using bioassay systems with locust (*Schistocerca gregaria*), we examined the effect of elevated CO_2 (control, 350; 650; 1000 ppm CO_2) on efficacy of strains of all three species and used crickets (*Acheta domesticus*) to examine interacting conditions of elevated temperature and CO_2 at two relative humidities (25-35°C; 350, 1000 ppm CO_2; 96, 98 and >99% RH) on efficacy of a strain of *B. bassiana* for the first time. The 3-way interacting factors had a significant effect on growth of the strains of all three species, especially at 35-37°C and 0.96-0.98 a_w and 1000 ppm CO_2. Under these conditions, only one strain of *B. bassiana* and *M. anisopliae* was able to grow at a reduced rate as compared to the controls. No strain of *I. farinosa* was able to grow at 35-37°C either in normal air or in elevated CO_2 at 0.995-0.96 a_w showing a high level of sensitivity to these interacting factors. Sporulation of the three strains of each species was also significantly affected by these three-way environmental interactions. There were some intra-strain differences and in most cases for the three species, water stress (0.98-0.96 a_w) at 35-37°C and 1000 ppm CO_2 resulting in either no sporulation or no growth. One strain of *M. anisopliae* (Ma 29) was particularly tolerant at 0.96 a_w at 37°C and 1000 ppm CO_2. Bioassays with the *S. gregaria* showed when CO_2 was elevated from 350 to 650 and to 1000 ppm, the relative virulence of two strains of each species was reduced over a 6-day temporal study. Further studies with *B. bassiana* in a detailed bioassay using crickets under three way abiotic interactions (25-35°C, 99-96% RH and 350 or 1000 ppm CO_2) showed that virulence was decreased with no efficacy occurring at 30-35°C and 1000 ppm CO_2 at 96% RH. This study suggests that climate change factors could have a profound impact on the efficacy of such biocontrol agents and thus have major implications for pest control using such approaches.

Key words: Water stress, temperature, elevated CO_2, growth, sporulation, entomopathogenic fungi, pest control.

INTRODUCTION

There has been significant interest in the impact that abiotic change scenarios may have on economically important crops and the associated pests and diseases. Indeed most climate change models suggest that there

will be a marked decrease in summer precipitation and increases in temperature, which will result in related drought stress episodes interspersed with periods of unusually high precipitation depending on the part of the world (European Commission, 2007; Solomon et al., 2009; Chalcraft, 2009). The environment in which crops will be grown in the next 10-20 years may change markedly with atmospheric CO_2 concentrations expected to double or triple. Due to this increase and that of other greenhouse gases, the global temperature is expected to increase by between +2 to 5°C (Dawson and Spannagle, 2008; Gray, 2009). The effects have been predicted to be detrimental or advantageous depending on the region. For example, in northern Europe, a mean temperature increase of 3 to 4.5°C, with a significant increase in precipitation of 30-40% was predicted (IPCC, 2007). Southern Europe is expected to be a hot spot for extreme temperature and drought stress which may have impacts on crop yield and pests and fungal diseases (Maistrello et al., 2006). Similar hot spots have been predicted to occur in parts of sub-Saharan Africa, South America and parts of Asia (IPCC, 2007). A recent study has predicted that, on a global scale, pests and diseases are moving to the poles at the rate of 3-4 km/year (Bebber et al., 2013). The possible implications this may have for the development of strategies to minimize pest and fungal pathogens of staple crops, especially the use of biological control agents has not been addressed.

The use of entomopathogenic fungi for pest management, especially as part of an Integrated Pest Management (IPM) strategy, combined with cultural and other methods has increased because of the reduction in available chemical control measures (Ansari et al., 2011; Beris et al., 2012; Pelizza et al., 2012; Svedese et al., 2012). It has however been previously shown that both temperature and water availability (water activity, a_w) are bottlenecks in the efficacy of entomopathogenic fungi against pests (Magan, 2007). High environmental temperatures and drought may reduce the growth rates of entomopathogens. For example, a recent study by Borisade and Magan (2014) screened the environmental tolerance of strains of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria farinosa* strains (5-6 for each), it showed that very few strains were able to tolerate elevated temperatures (35°C) and water stress (0.96-0.94 a_w). Strains of *M. anisopliae* were the most tolerant, regardless of the region of isolation. The study shows that sporulation of the strains of these fungi was significantly affected, with implications for secondary infection under interacting environmental conditions. The study, however, took no account of the influence of elevated CO_2. Thus, information is required on the impact that interactions of a_w, temperature and elevated CO_2 may have on growth, sporulation and insect infection by strains of these fungi.

The objective of the present study was to examine the effect of interactions between a_w, temperature and elevated CO_2 on (a) growth and (b) sporulation of three strains each of *B. bassiana*, *M. anisopliae* and *I. farinosa*. Bioassays were subsequently carried out with two strains of each entomogenous fungal species in bioassays with *S. gregaria* to compare the effect of temperature and equilibrium relative humidity (ERH) changes on virulence of the spores. Further efficacy of three way interactions of elevated CO_2 (1000 ppm), temperature (25-35°C) and ERH (>99%-96%) on virulence was assessed using *A. domestica*. The cricket was chosen for this bioassay because it survived the degree of abiotic stress factors being examined in the absence of fungal inoculum and is a useful bioassay system to examine the impact of the abiotic change scenarios on efficacy of such entomopathogenic biocontrol agents.

EXPERIMENTAL PROCEDURES

Source of fungal strains

Table 1 lists the species and strains examined in this study. The *B. bassiana* and *M. anisopliae* strains were kindly provided by the International Institute of Tropical Agriculture (IITA), Republic du Benin, West Africa, Prof T. Butt (University of Swansea, U.K.) and Dr D. Chandler (Warwick University, U.K.). The *I. farinosa* isolates with previously reported potential virulence against insects were kindly provided by the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Richard A. Humber, Insect Mycologist and Curator/ARSEF; USDA-ARS Biological Integrated Pest Management Research Unit, Robert W. Holley Centre for Agriculture and Health, Ithaca, NY 14853, USA).

Media preparation, inoculation and incubation

Sabouraud Dextrose Agar (Oxoid Ltd; 0.995 a_w) was modified with glycerol to 0.98, 0.96 and 0.94 a_w (Chen and Mujumdar, 2009). The accuracy of the modifications was confirmed using an Aqualab 3TE instrument (Decagon, Pullman, WA, USA) and found to be within ± 0.005 of the target a_w.

The agar media in 9 cm Petri plates were centrally inoculated with a 5 µl spore suspension containing about 1.4 x 10^7 spores ml^{-1} (Hallsworth and Magan, 1999). The replicates of each treatment strain and species were placed in separate polyethylene chambers (25 L capacity) together with 2 x 500 ml glycerol/water solution of the same a_w as the treatment plates. These were incubated at 25, 30, 35 and 37°C.

Elevated CO_2 exposure system

The polyethylene chambers containing the treatments and replicates were flushed with the required CO_2 concentrations of atmospheric

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Table 1. Origin of the fungal isolates used in this study.

Species	Isolate/strain	Host/Source	Country
B. bassiana	BB 315	Soil	Benin Republic
B. bassiana	BB 432.99	Unknown	United Kingdom
B. bassiana	BB 776.05	Coleoptera	United Kingdom
M. anisopliae	Ma 29	Soil	Benin Republic
M. anisopliae	Ma 27	Coleoptera	Benin Republic
M. anisopliae	V275	Unknown	United Kingdom
I. farinosa	IF 5081	Aleyrodidae	Pakistan
I. farinosa	IF 5676	Scutelleridae	Turkey
I. fumosorosea	PF 790.05	Unknown	United Kingdom

air (350 ppm), synthetic air with the CO_2 concentration specification of 1000 ppm provided by the British Oxygen Company (BOC). The chambers were flushed every two days with the required CO_2 concentrations at 2 $Lmin^{-1}$ to replace three times the volume of the incubation chamber. This was done immediately after growth measurements were made. The synthetic air moisture was controlled by inserting a bubbling device, containing a_w controlled solution of water/glycerol, just prior to the inlet valve when flushing each treatment chamber. The valves were sealed and the chambers incubated at the target treatment temperatures.

Growth and sporulation quantification

The diameter of the colonies was measured in two directions perpendicular to each other every two days for a period of up to 14 days. The diametric growth rate (mm day^{-1}) of the colonies of each strain and species under the different sets of interacting environmental conditions were computed by plotting the diameter of the colonies against time. Regression lines were made of the time points which represented the linear phase of the growth curves. These were used to determine the relative growth rates under different interacting conditions (Borisade and Magan, 2014). Experiments were carried out with three replicates per treatment.

The 14 day old cultures of all treatments were harvested by flooding the surface of the agar plate with 2 x 10 ml sterile water containing 0.05% Tween 80 and agitating with a sterile glass rod. Spore suspensions were poured into 25 ml universal bottles and centrifuged at 2500 rpm for 15 min. The supernatant was decanted and the spore concentrate made up to 1 ml. The spores were counted using a Neubauer haemocytometer (Abdel-Hadi et al., 2009). The sporulation data is presented as numbers of spores per ml per cm^2 of colony.

Bioassays with *S. gregaria* and *A. domesticus*

Nymphs of desert locust, *S. gregaria* and house crickets, *A. domesticus* (age of nymphs not known) were purchased from a commercial insectary in the UK. The locusts were fed with green leaves while *A. domesticus* was fed with rice bran in insect rearing cages at 25°C and 85% relative humidity for 24 h before being used for bioassays.

Fungal culture and inoculum preparation for bioassays

Two strains each of *B. bassiana* (BB 315 and BB 776.05), *I. farinosa* or *I. fumosorosea* (ARSEF 5081, IF 790.05) and *M. anisopliae* (Ma 275.86 DC, and V275) were cultured on Sabouraud Dextrose Agar (SDA) plates, sealed with parafilm and kept in sealable nylon bags. The plates were incubated at 25°C in the dark. Spores from 14 days old SDA plates were harvested by flooding the plate with 10 ml sterile reverse osmosis water and the spores gently dislodged with a glass rod. No surfactant was used. The spore suspension was transferred into 15 ml disposable Eppendorf centrifuge tubes and centrifuged at 3500 rpm for 20 min to concentrate the spores. The spores were thereafter made up to 1 ml, serial dilutions were made and the spore concentration was determined with a haemocytometer. A single concentration bioassay was done with 1.0 x 10^5 conidia ml^{-1} of each isolate as the inoculum.

All experiments were performed using 15 cm Petri-dishes with a 1 cm^2 cut lid and the cut area lined with muslin cloth held in place with masking tape to prevent the insects escaping. A Whatman No. 1 filter paper was placed flat in the bottom of each Petri dish to serve as an absorbent material for excess water in the inoculum preparation. Ten (10) nymphs of either *A. domestica* or *S. gregaria* were separately placed in each Petri-dish. Each set-up consisted of triplicate plates and a control. 1 ml of spore suspension containing about 1 x10^5 spores per ml was sprayed directly on the nymphs in each plate. The bio-assay was a single dose experiment. The dishes were arranged inside a plastic box measuring 30 x 30 x 25 cm^3 and a tight fitting lid. 500 ml of either deionized water or saturated salt solutions in glass beakers were placed in each box to modify the ERH (99-96% ± 1% ERH) at different temperatures (25-35°C) (Lewis, 1976; Charles and David, 1992). The mortality of the insects was observed daily for 6 days.

Effect of elevated CO_2 and relative humidity on fungal virulence using *A. domesticus*

The house cricket was used in a model study to evaluate the effect of interacting climate change factors on efficacy of fungi for management of insect pests of agricultural crops.

Two strains each of *B. bassiana* (BB 315, BB 776.05), *I. farinosa* or *I. fumosorosea* (IF 5081, PF 790) and *M. anisopliae* (Ma 275.86, V275) were used to investigate the effect of elevated CO_2 (650 and 1000 ppm carbon dioxide-air mixture) on spore virulence at 100% relative humidity and 25°C. The experimental set-up consisted of 3 replicates of 10 insects per jar covered with muslin. The inoculum (0.5 ml of 1.0 x 10^5 conidia ml^{-1}) was sprayed on the insects through a slit cut on the muslin. Thereafter, the slit was covered with a masking tape. The treatment for each isolate consisted of insects + inoculum + CO_2 (air, 650 or 1000 ppm). The outlet tap of the box was kept open after the jars were arranged in the box and the lid tightly placed while the inlet tap was connected to a CO_2-air mixture

ANOVA Table 1. Effect of abiotic interactions on sporulation of 3 strains of *Beauveria bassiana.*

Tests of between-subjects effects					
Dependent variable: Log_{10} (spore cm^{-2} + 1)					
Source of variations	**SS**	**DF**	**MS**	**F**	**Sig.**
CO_2 atmosphere	228.544	1	228.544	91750.655	sig
Temperature	320.821	3	106.940	42931.988	sig
Water activity (a_w)	143.901	2	71.950	28885.005	sig
Isolates x CO_2 atmosphere	122.546	2	61.273	24598.513	sig
Isolates x Temperature	44.692	6	7.449	2990.305	sig
Isolates x a_w	27.782	4	6.946	2788.345	sig
Isolates x CO_2 atmosphere x Temperature	38.782	6	6.464	2594.897	sig
Isolates x CO_2 atmosphere x a_w	5.689	4	1.422	570.931	sig
Isolates x Temperature x a_w	107.828	12	8.986	3607.362	sig
Isolates x CO_2 atmosphere x Temperature x a_w	62.207	12	5.184	2081.133	sig
Total	3134.049	216			
Corrected Total	1325.433	215			
a. R Squared = 1.000 (Adjusted R Squared = 1.000)					

MS= Mean square, SS= sum of squares, DF= degree of freedom, Sig. = significant (P<0.05).

ANOVA Table 2: Effect of abiotic interactions on sporulation of 3 strains of *M. anisopliae*.

Tests of between-subjects effects					
Dependent variable: Log_{10} (Spore cm^{-2} + 1)					
Source of variations	**SS**	**DF**	**MS**	**F**	**Sig.**
Isolates	277.665	2	138.832	103472.858	sig
CO_2 atmosphere	324.579	1	324.579	241911.345	sig
Temperature	291.516	3	97.172	72423.180	sig
a_w	44.113	2	22.057	16438.977	sig
Isolates x CO_2 atmosphere	35.044	2	17.522	13059.280	sig
Isolates x Temperature	123.604	6	20.601	15353.856	sig
Isolates x a_w	98.132	4	24.533	18284.575	sig
Isolates x CO_2 atmosphere x Temperature	221.603	6	36.934	27527.048	sig
Isolates x CO_2 atmosphere x a_w	28.613	4	7.153	5331.411	sig
Isolates x Temperature x a_w	75.212	12	6.268	4671.325	sig
CO_2 atmosphere x Temperature x a_w	8.974	6	1.496	1114.719	sig
Isolates x CO_2 atmosphere x Temperature x a_w	59.107	12	4.926	3671.081	sig
Total	4139.254	216			
Corrected total	1813.954	215			
a. R Squared = 1.000 (Adjusted R Squared = 1.000)					

source. The gas flow rate was adjusted to 5 L min^{-1} for 15 min after which the inlet tap was closed followed by the outlet. The set up was maintained at 25°C and daily cumulative insect mortality was recorded for 6 days. The box was flushed with the treatment CO_2 level every 24-h period.

The experiments included controls which consisted of (a) Insects + normal air, (b) Insects + CO_2 (650 ppm) and (c) Insects + CO_2 (1000 ppm). The controls (Insects + normal air) were compared with the 2 different CO_2 levels (650 and 1000 ppm) to evaluate toxicity of elevated CO_2 alone to the insects.

The 2 different elevated CO_2 was found to not be lethal to the insects and mortality was comparable with that in normal air. Overall, there was less than 10% mortality in normal air and elevated CO_2 conditions during the period of the study. During the six days observation period, there was no mortality in the controls at 650 ppm while <10% mortality was recorded both in the control with normal air and elevated CO_2 at 1000 ppm.

Data analysis

Growth and sporulation data were log transformed and checked for normality. Equality of error variances was confirmed with Levene's test and thereafter analysed using ANOVA procedure and different means were compared using the Tukeys test at P = 0.05. The data was back transformed for graphical presentation. All analyses were done using the statistical package, IBM SPSS Statistics 20 (ANOVA Tables 1 to 3).

ANOVA Table 3. Effect of abiotic interactions on sporulation of 3 strains of *Isaria farinose.*

Tests of between-subjects effects Dependent variable: Log_{10} (Spore cm^{-2} + 1)					
Source of variations	**SS**	**DF**	**MS**	**F**	**Sig.**
Isolates	194.000	2	97.000	97.925	sig
CO_2 atmosphere	19.548	1	19.548	19.734	sig
Temperature	283.209	3	94.403	95.303	sig
a_w	150.225	2	75.113	75.829	sig
Isolates x CO_2 atmosphere	11.645	2	5.822	5.878	sig
Isolates x Temperature	111.009	6	18.501	18.678	sig
Isolates x a_w	26.785	4	6.696	6.760	sig
Isolates x CO_2 atmosphere x Temperature	16.476	6	2.746	2.772	sig
Isolates x CO_2 atmosphere x a_w	2.147	2	1.073	1.084	sig
Isolates x Temperature x a_w	33.654	6	5.609	5.663	sig
Isolates x CO_2 atmosphere x Temperature x a_w	37.596	4	9.399	9.489	sig
Error	104.008	105	.991		
Total	2016.216	153			
Corrected total	1028.612	152			
a. R squared = 0.899 (Adjusted R squared = 0.854)					

For *S. gregaria*, a daily cumulative mortality was recorded and the mean percentage survival was calculated (Keyser et al., 2014). The daily cumulative mortality was similarly recorded for *A. domesticus* and a graph of the values of corrected mean percentage mortality was plotted against time. LT_{50} was calculated from the regression equation of the mortality against time.

RESULTS

Effect of a_w x temperature x elevated CO_2 on growth of the strains of entomopathogens

Figures 1, 2 and 3 compare the effect of these interacting factors on the relative growth rates of the three strains of each species examined (*B. bassiana*, *M. anisopliae* and *I. farinose*, respectively). For *B. bassiana*, with freely available water (0.995 a_w) all the strains could grow over the 25-37°C range in air. As temperature was increased to 35°C, there was a significant decrease in growth rate, especially at 0.98 a_w. With drier conditions (0.96 a_w), BB 432.99 could only grow at 25°C. Interactions between a_w, temperature and elevated CO_2 affected the growth rate of the strains, especially at 0.995 a_w and 25-30°C. While growth was often slower than in atmospheric air, one *B. bassiana* strain (BB 315) was able to grow effectively at 0.98 and 0.96 a_w at 35°C in the presence of elevated CO_2. None of the strains could grow at 37°C and 0.96 a_w, regardless of CO_2 treatments.

For *M. anisopliae* there was a marked difference in growth between one of the strains (Ma29) and the others in both normal atmospheric air and elevated CO_2, regardless of a_w and temperature treatment. Interestingly, in elevated CO_2, the *M. anisopliae* strain Ma29 was able to grow better than in air at both 0.995 and 0.98 a_w across the temperature range tested. The other two strains were more sensitive to the three way interacting conditions and unable to grow at 37°C regardless of a_w regime imposed.

I. farinosa strains on the other hand were less tolerant; only ARSEF 5676 and IF 790.05 could grow at 25°C and three treatment a_w levels (0.995, 0.98 and 0.96) under elevated CO_2 whereas, under normal air conditions the temperature windows for growth of the 2 strains was 25-30°C at 0.995 and 0.98 a_w, respectively. In contrast, ARSEF 5081 could grow at 35°C and 0.96 a_w under elevated CO_2 while no growth occurred under such conditions in the normal air.

Effect of a_w, temperature and elevated CO_2 on sporulation of the entomopathogens

The effect of the interactions of temperature and a_w on sporulation of the strains of *B. bassiana*, *M. anisopliae* and *Isaria* strains are shown in Tables 2, 3 and 4. The optimum temperature for sporulation varied with a_w and was also strain dependent. The *B. bassiana* strain BB 315 produced significantly higher numbers of spores than BB 432.99 and BB 776.05 at higher temperatures (35 and 37°C) and 0.995 a_w under normal atmospheric conditions. In contrast, the strain BB 432.99 produced significantly higher spore numbers under elevated CO_2 at 0.995 a_w and 25, 30 and 35°C. The *B. bassiana* strain BB 315 was unusual as it could grow under elevated CO_2 at 35°C and 0.96 a_w but not in normal air. However, it could not sporulate at 0.98 a_w and 35°C under elevated CO_2.

One of the three *M. anisopliae* strains (V275) could not

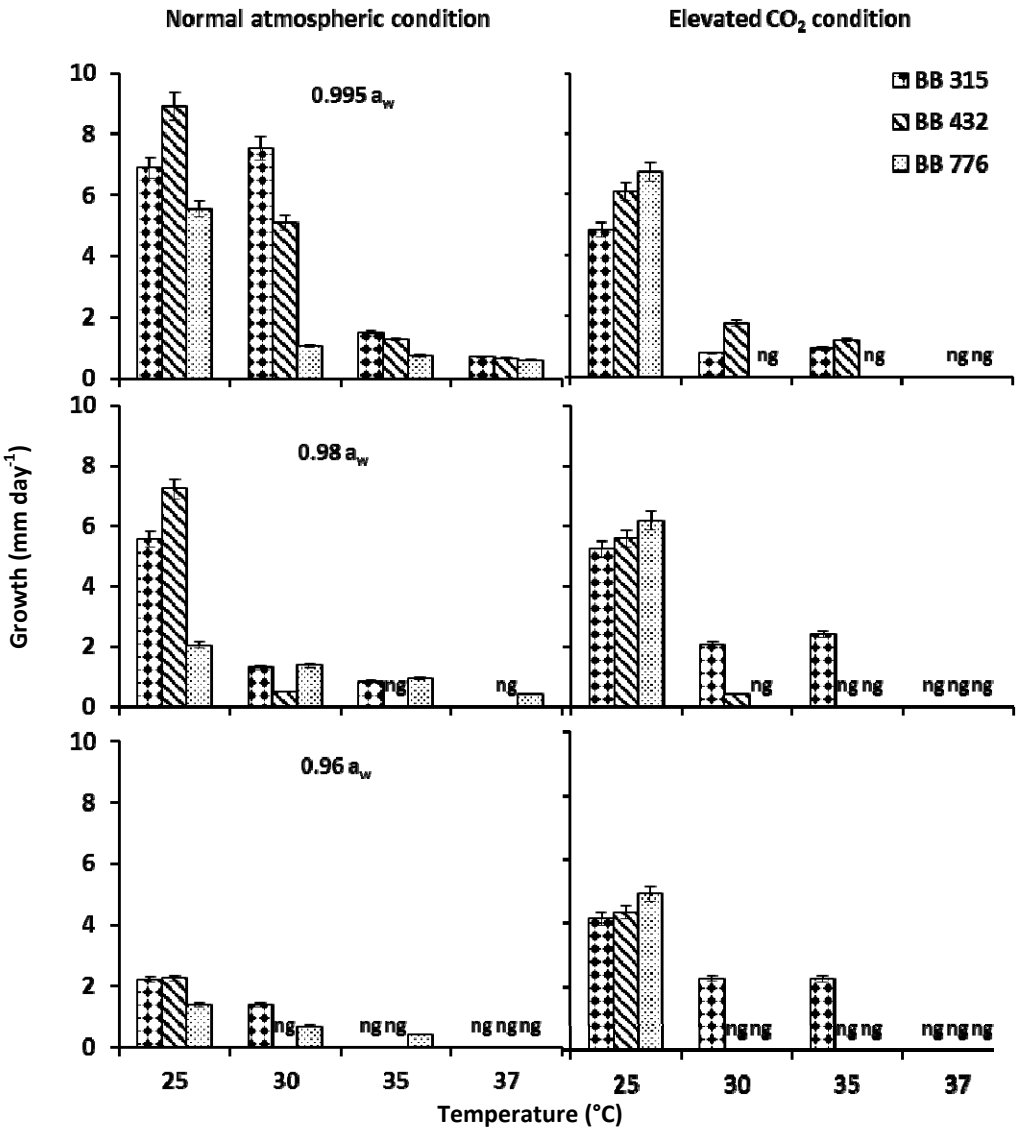

Figure 1. Comparisons of effect of temperature and a_w on growth of *B. bassiana* under normal atmospheric air composition and elevated CO_2 conditions. Ng = no growth.

sporulate under interacting abiotic stress conditions (0.995 a_w, 30 and 35°C; 0.98 a_w and 30°C) in elevated CO_2 atmosphere conditions. In some cases, no sporulation occurred under elevated CO_2 even at high water activity (0.995 a_w) and 30°C in *M. anisopliae* strains. Thus, In elevated CO_2, the combined stresses appeared to have a detrimental effect on sporulation with significantly less or no spore production occurring, even when water was freely available (0.995 a_w) and moderate temperature of 30°C.

Overall, the *Isaria* strains were very sensitive to changes in the three interacting factors with sporulation significantly affected by elevated CO_2, especially at elevated temperatures. The *Isaria* strains IF 5081 and IF 5676 could not sporulate at 0.98 a_w x 37°C and 0.96 a_w x 25°C, respectively under elevated CO_2 whereas sporulation was possible under such conditions in the normal air.

Relative virulence of the entomopathogens in normal air and elevated CO_2 (650 and 1000 ppm) against *S. gregaria*

Figure 4 compares the effect of normal air, 650 and 1000 ppm CO_2 on the survival of the desert locust, *S. gregaria* under 100% RH conditions on the relative virulence of 2 strains each of *B. bassiana* (BB 315, BB 776.05), *M. anisopliae* (275.86DC, V275), and one strain each of *I. farinosa* (ARSEF 5081) and *I. fumosorosea* (790.05) over

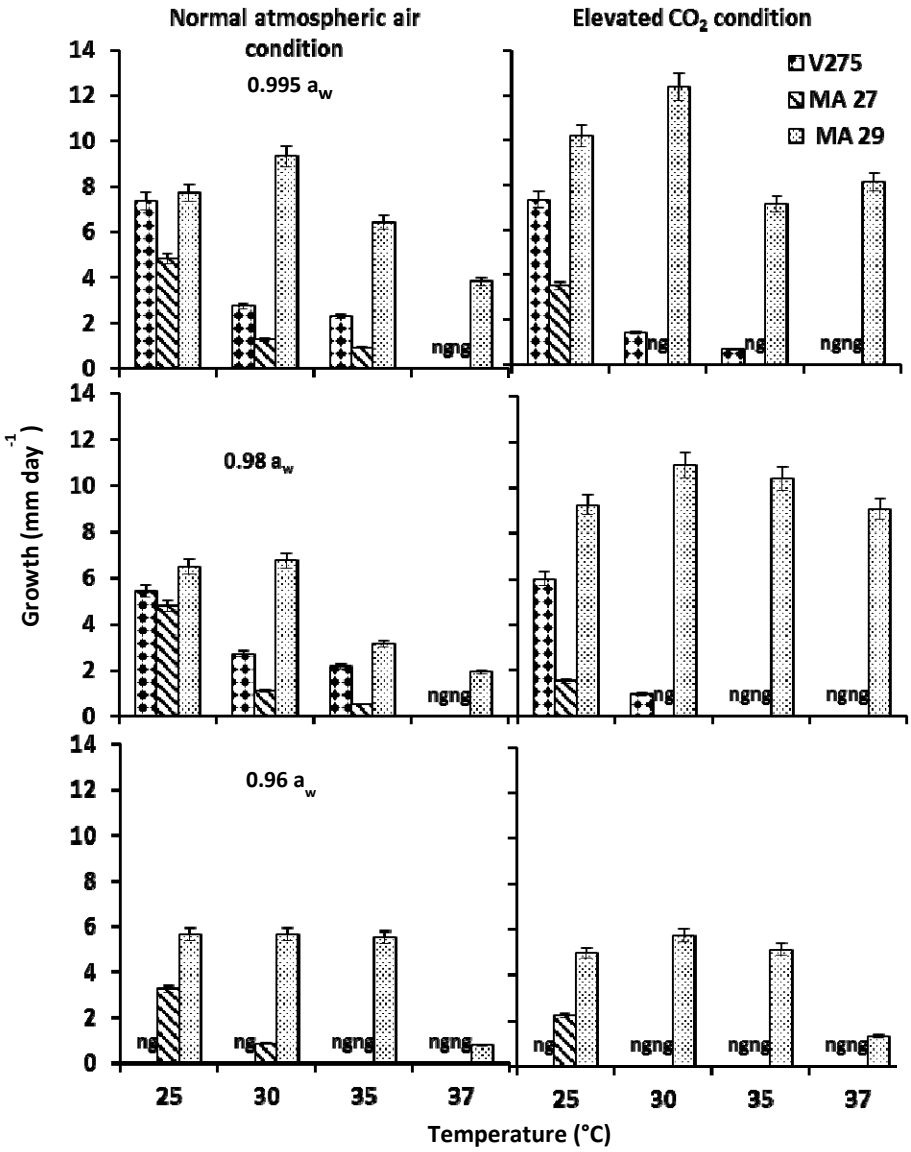

Figure 2. Comparisons of effect of temperature x a_w on growth of *M. anisopliae* under normal atmospheric air composition and elevated CO_2 conditions.

six days.

Under ambient atmospheric air composition, a strain of *B. bassiana* (776.05) showed relatively weak pathogenicity to *S. gregaria.* After 5 days of application of the spores to *S. gregaria*, the data showed 27% of the insects survived the treatment whereas, 93-100% mortality was recorded in the treatment with other strains. *I. fumosorosea* caused 100% mortality after 4 days and this was the most virulent of all the tested strains.

Under elevated CO_2 (650, 1000 ppm CO_2) more of the insects survived the treatments. For example, the virulence of the weakly pathogenic strain (776.05) was further affected such that 60 and 93% of the inoculated insects survived after 5 days at 650 and 1000 ppm, respectively. A similar reduction in virulence by elevated CO_2 was observed in all the strains. 20% of the inoculated insects survived inoculation with spores of the most pathogenic isolate, *I. fumosorosea* 790.05 after 4 days with 7% surviving after 5 days. With the exception of the *Isaria* strain (ARSEF 5081) where 100% mortality of the inoculated insects was recorded after 6 days, about 6-13% of the insects survived after inoculation with the other strains at 1000 ppm.

Relative virulence of *B. bassiana* (BB 315) to *A. domesticus* under different elevated temperature x CO_2 x relative humidity conditions

Figure 5 shows the changes in virulence of *B. bassiana*

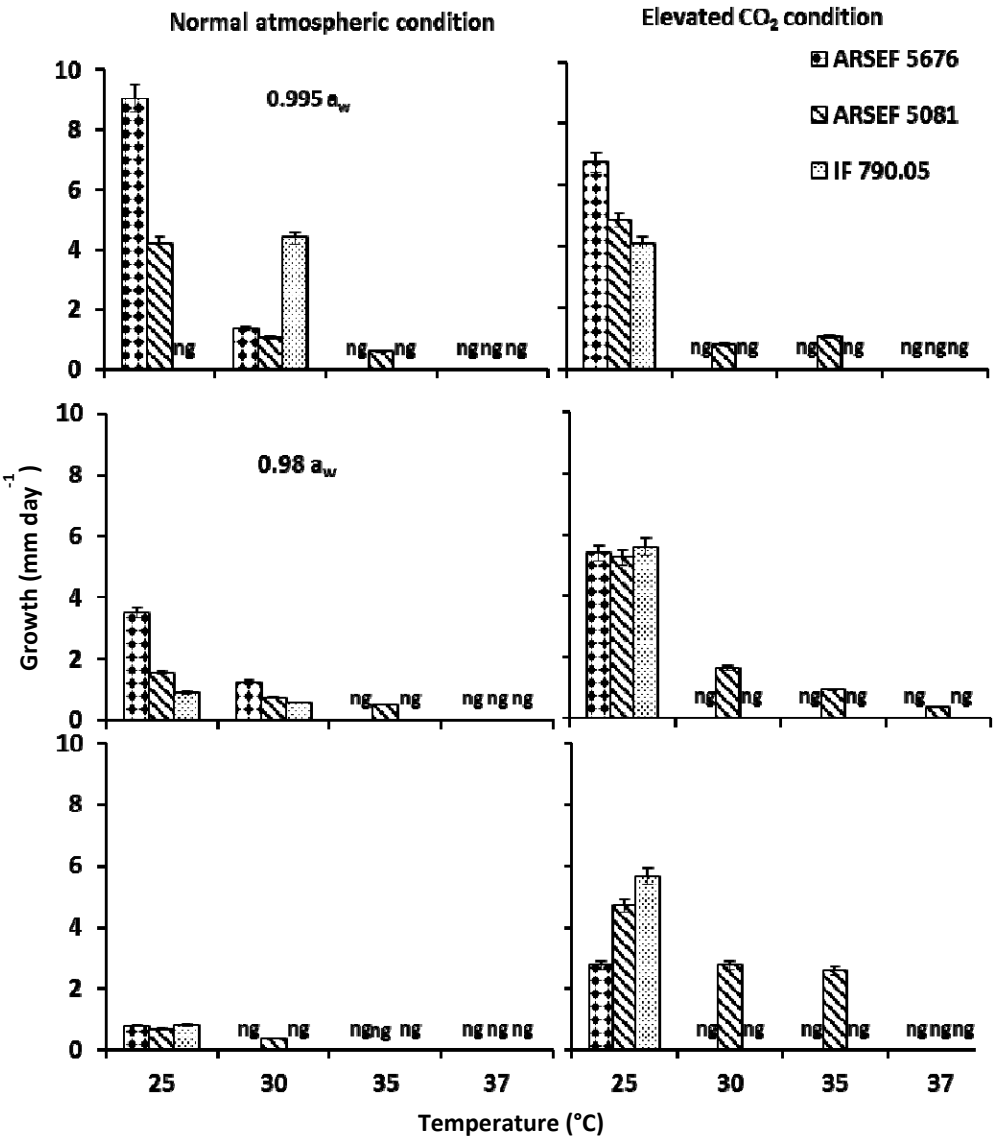

Figure 3. Comparisons of effect of temperature x water activity (a_w) on growth of *I. farinosa* under normal atmospheric air composition and elevated CO_2 conditions.

(BB 315) under normal air and 1000 ppm CO_2 and elevated temperatures and different relative humidities. There was an extension in the time (LT_{50}) required for this entomopathogen to cause mortality of the insect populations. At 25°C and >99% ERH, the LT_{50} of the strain was 5.9 days. Under a stress condition (96% ERH and 25°C), this increased to 6.5 days. Higher LT_{50} values were recorded as the interacting climate change factors became increasingly severe. The LT_{50} of the fungi to *A. domesticus* at 35°C and 96% ERH (9.3 days) was more than double of that at 35°C and >99% ERH.

Elevation of CO_2 concentration to 1000 ppm significantly increased the LT_{50} regardless of temperature and ERH. For instance, 30°C and >99% RH was optimum for virulence of the strain (lowest LT_{50}= 4.4 days) in ambient air. In the presence of 1000 ppm CO_2 and >99% RH, the LT_{50} at the same temperature increased to 13.1 days. Under drier conditions (96% RH) and 30-35°C, all the inoculated insects survived showing that under these conditions, the biocontrol entomopathogen was ineffective over the experimental period.

DISCUSSION

This is the first study that examine the impact of interacting climate change conditions (temperature, a_w and elevated CO_2) on the growth and sporulation of entomopathogens. This study has shown that these

Table 2. Comparison of the effect of interactions of temperature (25-37°C) and water activity (0.995 - 0.96 a_w) on sporulation (log_{10} spore cm^{-2}) of three strains of *B. bassiana* under ambient CO_2 (350 ppm CO_2) and elevated CO_2 (1000 ppm).

Fungal strain	0.995 a_w			0.98 a_w			0.96 a_w		
	BB315	BB 432.99	BB 776.05	BB315	BB 432.99	BB 776.05	BB315	BB 432.99	BB 776.05
25°C									
Ambient	4.77[b]	3.37[b]	5.07[a]	6.25[a]	3.76[b]	4.61[a]	5.77[a]	4.75[a]	4.75[a]
1000 ppm CO_2	5.62[a]	5.76[a]	3.63[b]	3.37[b]	4.76[a]	3.88[b]	3.17[b]	4.05[b]	3.57[b]
30°C									
Ambient	5.09[a]	3.86[b]	6.03[a]	5.91[a]	5.92[a]	5.08[a]	4.95[a]	NG[c]	5.38[a]
1000 ppm CO_2	3.93[b]	4.80[a]	NG[c]	4.58[b]	3.89[b]	NG[c]	3.48[b]	NG[c]	NG[c]
35°C									
Ambient	6.23[a]	4.69[a]	6.11[a]	5.43[a]	NG[c]	5.18[a]	NG[c]	NG[c]	5.14[a]
1000 ppm CO_2	3.16[b]	4.17[b]	NG[c]	NS[c]	NG[c]	NG[c]	NS[c]	NG[c]	NG[c]
37°C									
Ambient	5.67[a]	4.98[a]	5.25[a]	NG[c]	NG[c]	5.13[a]	NG[c]	NG[c]	NG[c]
1000 ppm CO_2	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]

The table compares effects of atmospheric CO_2 within each level of combination of the other factors (temperature and water activity) shown. These tests are based on the linearly independent pairwise comparisons among the estimated marginal means. For each strain and within the same level of interaction (temperature and a_w), values followed by different letters are significantly different ($P<0.05$) at different a_w and temperature levels for ambient vs. 1000 ppm CO_2. NG = No growth, NS = there was growth but no sporulation

interacting abiotic factors profoundly influenced the growth and sporulation of the fungal species and strains examined. Elevated CO_2 influenced the temperature range for growth and changed the set of conditions for optimum growth and sporulation in all the strains. Optimal growth conditions for all the strains were between 25-35°C and 0.995-0.98 a_w under unmodified CO_2 and elevated CO_2 conditions.

No comparisons can be made with previous data except where a_w and temperature conditions on growth and sporulation were considered (Hallsworth and Magan, 1996; Borisade and Magan, 2014). Indeed, Borisade and Magan (2014) showed that elevated temperatures to simulate those under climate change conditions when interacting with drought stress significantly influenced the ability of strains of *B. bassiana*, *M. anisopliae* and *I. farinosa* to grow. In these studies, only a few strains were able to tolerate 35-37°C and drought stress (0.94-0.96 a_w) and these were one strain each of latter two species. The source of the strains did not influence tolerance to elevated temperatures and drought stress.

In the present study, the interaction with elevated CO_2 suggests that further modulation of growth may occur. It may be that some strains are able to change their growth morphology to tolerate such conditions. For example, *I. farinosa* (ARSEF 5081) was able to tolerate elevated atmospheric CO_2 by changing its growth morphology under the specific temperature and a_w conditions in the present study. In earlier studies (Alves et al., 2002), *B. bassiana* was found to develop yeast like cells on SDA in the presence of the imposed ionic solute stress using NaCl. Yeast-like cell formation in entomopathogenic fungi is an adaptation for survival in the insect haemolymph (which could be rich in CO_2 and solutes). Yeast-like cells of *B. bassiana* have been reported to occur on media when the condition of the media mimics the haemolymph of insects (Alves et al., 2002). This may be responsible for the higher growth at lower a_w and elevated CO_2 in some strains of the species examined in the present study.

Overall, the strain Ma 29 of *M. anisopliae* showed a relatively higher resilience to the interactions of the extremes of the abiotic stress factors, as the temperature profile for growth (25-37°C) was not altered under elevated CO_2 and when combined with water stress.

It appeared that elevated CO_2 significantly influenced the ability for conidial production and

Table 3. Comparison of the effect of interactions of temperature (25-37°C) and water activity (0.995 - 0.96 a_w) on sporulation (log_{10} spore cm^{-2}) of three strains of *M. anisopliae* under ambient (350 ppm CO_2) and elevated CO_2 (1000 ppm CO_2).

Fungal strain	0.995 a_w			0.98 a_w			0.96 a_w		
	Ma 29	Ma 27	V275	Ma 29	Ma 27	V275	Ma 29	Ma 27	Ma V275
25°C									
Ambient	4.62[a]	7.05[a]	6.03[a]	4.12[a]	7.04[a]	5.25[a]	4.23[a]	7.42[a]	NG[c]
1000 ppm CO_2	3.98[b]	6.18[b]	5.54[b]	3.46[b]	5.97[b]	5.76[b]	4.14[b]	6.15[b]	NG[c]
30°C									
Ambient	4.29[a]	8.06[a]	5.78[a]	4.82[a]	8.38[a]	6.90[a]	7.19[a]	7.93[a]	NG[c]
1000 ppm CO_2	4.34[a]	NG[c]	NS[c]	4.71[b]	NG[c]	NS[c]	3.20[b]	NG[c]	NG[c]
35°C									
Ambient	4.09[a]	8.40[a]	4.73[a]	4.61[a]	8.90[a]	6.24[a]	4.59[a]	NG[c]	NG[c]
1000 ppm CO_2	3.37[b]	NG[c]	NS[c]	3.71[b]	NG[c]	NG[c]	3.64[c]	NG[c]	NG[c]
37°C									
Ambient	4.28[a]	NG[c]	NG[c]	6.25[a]	NG[c]	NG[c]	5.60[a]	NG[c]	NG[c]
1000 ppm CO_2	3.39[b]	NG[c]	NG[c]	3.67[b]	NG[c]	NG[c]	4.70[b]	NG[c]	NG[c]

The table compares effects of atmospheric CO_2 within each level of combination of the other factors (temperature and water activity) shown. These tests are based on the linearly independent pairwise comparisons among the estimated marginal means. For each strain and within the same level of interaction (temperature and a_w), values followed by different letters are significantly different (P<0.05) at different a_w and temperature levels for ambient vs. 1000 ppm CO_2. NG = No growth, NS = there was growth but no sporulation.

Table 4. Comparison of the effect of interactions of temperature (25-37°C) and water activity (0.995 - 0.96 a_w) on sporulation (log_{10} spore cm^{-2}) of three strains of *I. farinosa* in ambient (350 ppm CO_2) and under elevated CO_2 (1000 ppm).

Fungal strain	0.995 a_w			0.98 a_w			0.96 a_w		
	ARSE F 5081	ARSE F 5676	IF 790.05	ARSEF 5081	ARSEF 5676	IF 790.05	ARSEF 5081	ARSEF 5676	IF 790.05
25°C									
Ambient	4.17[a]	4.97[a]	3.71[b]	4.61[a]	4.73[b]	5.89[a]	4.75[a]	7.53[a]	6.32[a]
1000 ppm CO_2	3.55[b]	2.73[b]	3.44[b]	3.88[b]	7.01[a]	4.38[b]	3.57[b]	3.88[b]	NS[c]
30°C									
Ambient	5.72[a]	6.08[a]	4.40[a]	5.08[a]	6.21[a]	6.55[a]	7.33[a]	NG[c]	NG[c]
1000 ppm CO_2	4.80[b]	NG[c]	NG[c]	NG[c]	6.94[a]	NG[c]	4.22[b]	NG[c]	NG[c]
35°C									
Ambient	6.08[a]	NG[c]	4.93[a]	6.61[a]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]
1000 ppm CO_2	4.46[b]	NG[c]	NG[c]	6.59[a]	NG[c]	NG[c]	3.86[b]	NG[c]	NG[c]
37°C									
Ambient	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]
1000 ppm CO_2	NG[c]	NG[c]	NG[c]	**NS**[c]	NG[c]	NG[c]	NG[c]	NG[c]	NG[c]

The table compares effects of atmospheric CO_2 within each level of combination of the other factors (temperature and water activity) shown. These tests are based on the linearly independent pairwise comparisons among the estimated marginal means. For each strain and within the same level of interaction (temperature and a_w), values followed by different letters are significantly different (P<0.05) at different a_w and temperature levels for ambient vs. 1000 ppm CO_2. NG = No growth, NS = there was growth but no sporulation.

reduced the range of a_w and temperature over which this occurred. Previous studies have examined effects of temperature or a_w and temperature on sporulation indices but have not included the climate change factor and the concentrations used here of 1000 ppm CO_2 (Alves et al., 2002; Lord, 2009; Garza-Lopez et al., 2011; Borisade and Magan, 2014).

Overall, at least one strain of each entomogenous species showed considerable resilience to the effect of elevated CO_2 interactions at various temperatures and a_w

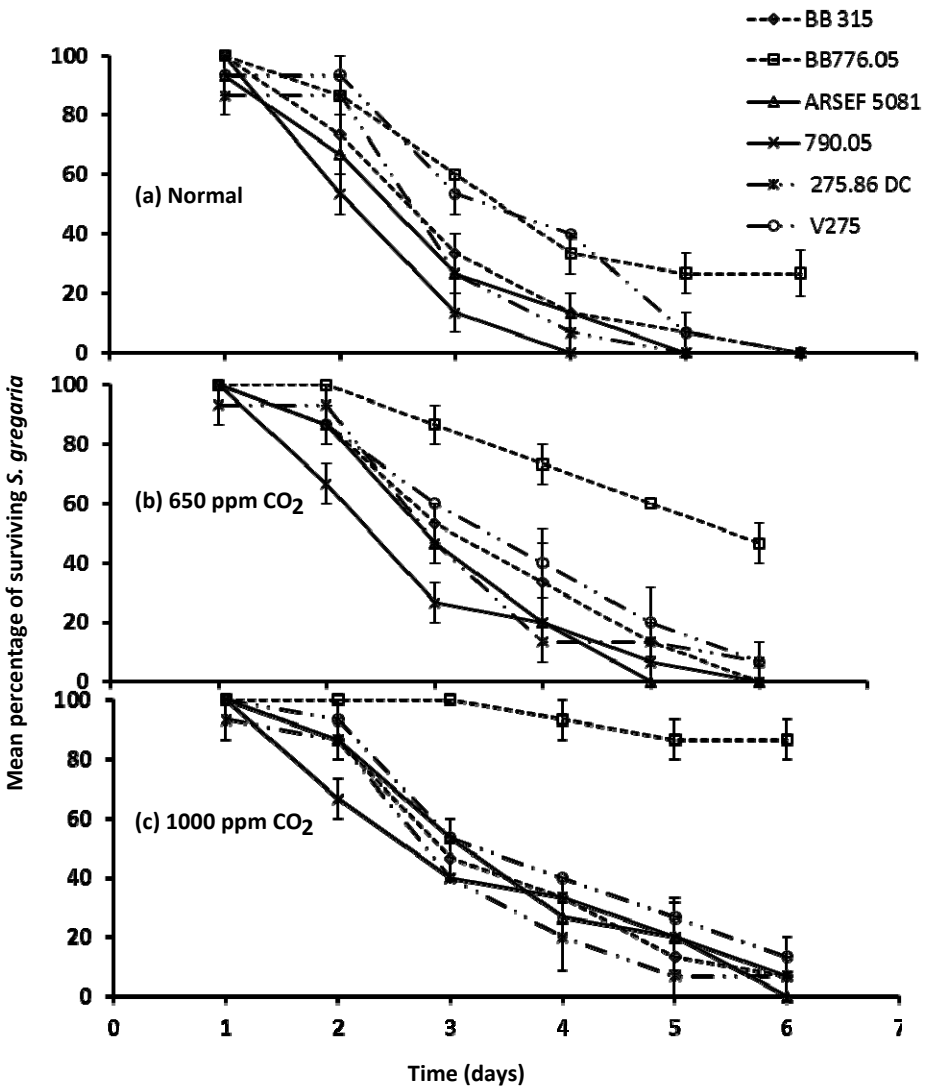

Figure 4. Mean percentage survival of desert locust (*Schistocerca gregaria*) separately exposed to entomogenous *Beauveria bassiana* (BB 315, BB 776.05), *Isaria farinosa* (ARSEF 5081), *Isaria fumosorosea* (790.05) and *Metarrhizium anisopliae* (275.86DC, V275) conidia at 3 different concentrations of CO_2 : (a) Normal air, 350 ppm, (b) 650 ppm, (c) 1000 ppm. Bars indicate standard error of the mean.

levels. However, elevated CO_2 significantly reduced sporulation potential. Currently, there is little information on the effect of CO_2 on sporulation of entomopathogenic fungi. The simultaneous effect of the interactions of the three abiotic stress factors under consideration in this study has not been previously reported for these strains.

In this study, *B. bassiana* (BB 315) was able to grow at 0.96 a_w x 35°C under elevated CO_2 but unable to sporulate. Similarly, *I. farinosa* (ARSEF 5676) could grow at 0.96 a_w x 25°C and *M. anisopliae* (V275) could grow at 0.995 a_w x 30/35°C under elevated CO_2, but no sporulation occurred. The observed loss of sporulation capabilities under a combination of abiotic stress factors can have a significant impact on the success of entomopathogenic strains in pest management system under climate change scenarios.

Previous studies have reported a decrease in conidial production in the presence of elevated CO_2 at concentrations significantly higher (10% CO_2) in *Aspergillus niger* and *Trichoderma viridis* (Desgranges and Durand, 1990). Similarly, Garza-Lopez et al. (2011) reported 85% decrease in conidial production in *B. bassiana* under 5% CO_2 enriched atmosphere while Lord (2009) reported increased germination lag times and subsequently, a decrease in both mycelial growth and sporulation.

The studies carried out with both *S. gregaria* and *A. domesticus* represent the first attempt to try and examine

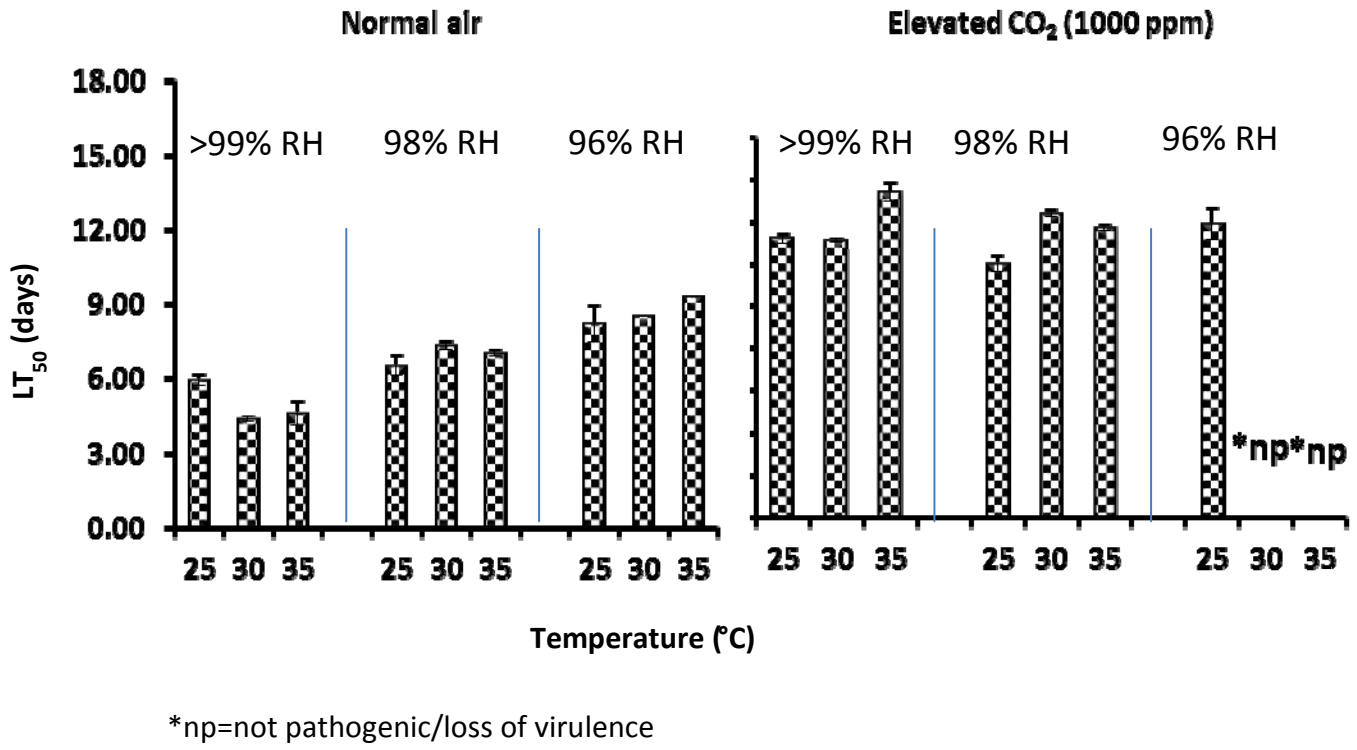

Figure 5. Relative virulence of *B. bassiana* (BB 315) to crickets (*Acheta domesticus,* Orthoptera: Gryllidae) under the influence of different interacting climate change factors (temperature x relative humidity x CO_2 concentrations). Bars indicate standard error of the mean.

the potential impact of temperature x elevated CO_2 on relative virulence of strains of these species in relation to factors which may simulate climate change scenarios. The results with two strains of each entomogenous species (*S. gregaria*) and that with *B. bassiana* in relation to the mortality of crickets have demonstrated that the virulence may be reduced, impacting on the level of control achieved.

Under a climate change scenario, the temperature is expected to rise by 2-4°C, the CO_2 to increase by up to three times existing levels (700-1000 ppm) under drought conditions. These combined factors may have very different impacts than one or two together and influence both pest and disease epidemics (Magan et al., 2007, 2011; Bebber et al., 2013). Thus, it is critical that this type of data are obtained and utilised to help in the selection of strains for use under the marginal conditions that may be present in a climate change scenario. This also suggests that IPM strategies may need to be significantly modified or formulations of entomogenous biopesticides need to be modified to facilitate consistent efficacy under such pressures. It may be that strains of entomogenous fungi need to be isolated from native agro-ecological zones or the formulation may require modification to enable efficacy to be relied upon under such abiotic change pressures. Indeed, less effective pest control in an IPM strategy may have a significant impact on food security of staple commodities, their quality and availability.

Conflict of interests

The authors did not declare any conflict of interest.

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