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In vitro selection of a fungal pathogen for use against *Diaphorina citri*

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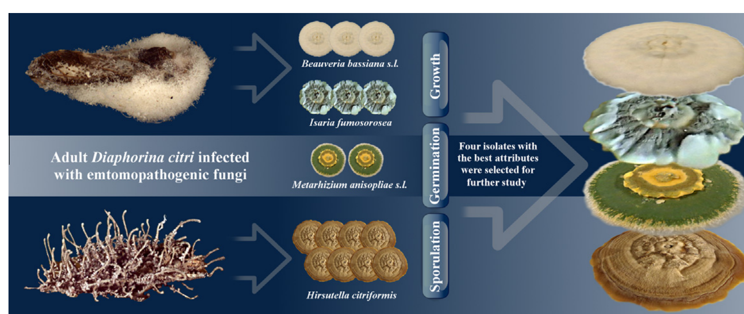
HIGHLIGHTS

- *Diaphorina citri* is an important pest that transmits the disease HLB in citrus.
- Entomopathogenic fungi may provide an important control strategy for *D. citri*.
- Growth, germination and sporulation of 16 isolates were assessed.
- Four isolates from different species showed greatest potential.

GRAPHICAL ABSTRACT



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ABSTRACT

The Asian citrus psyllid (ACP), *Diaphorina citri*, is an important pest of citrus, which transmits the disease Huanglongbing (HLB) and has had devastating effects on the citrus industry around the world. Control relies mainly on synthetic pesticides, but biological control using entomopathogenic fungi may provide an alternative strategy. We evaluated sixteen fungal isolates in total; three of *Beauveria bassiana*, two of *Metarhizium anisopliae*, three of *Isaria fumosorosea* (Group 1); and eight isolates of *Hirsutella citriformis* (Group 2). The *in vitro* growth, germination (after different incubation times) and sporulation were assessed for all isolates at four temperatures: 20, 25, 30 and 35 °C. Overall, the Group 1 isolates achieved the greatest growth, germination rate and sporulation compared with the *H. citriformis* isolates In Group 2. Among the Group 1 isolates, B1, M2 and I1 had the greatest performance in the biological attributes evaluated. Group 2 isolates were not competitive under *in vitro* assessment; however, they cannot be dismissed entirely until *in vivo* experiments are done as this species was the most common pathogen infecting ACP in the field.

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1. Introduction

Huanglongbing (HLB) is the most devastating disease of citrus worldwide; infected trees suffer reduced production and ultimately either die or become completely unproductive (Halbert and Manjunath, 2004; Bové, 2006; Manjunath et al., 2008, 2010; Tiwari et al., 2011; Mora-Aguilera et al., 2013). The bacterium *Candidatus* Liberibacter asiaticus has been reported as the causative agent in Mexico, and it is vectored by the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Lividae) (Bové, 2006; Tiwari et al., 2011).

HLB has been found in at least 10 Mexican States (Mora-Aguilera et al., 2013; SENASICA, 2013), and its control has been based on the elimination of HLB-infected trees, planting certified HLB-free trees and by reducing vector populations. Control of the vector, *D. citri*, has relied mainly on chemical insecticides to

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rapidly reduce the insect population, and therefore diminish the probability of HLB transmission (Brlansky et al., 2007; Qureshi and Stansly, 2008). However, continuous use of chemical insecticides can lead to negative impacts on the environment such as pollution of soil and water; it can also be detrimental to non-target insects and cause the emergence of secondary insect pests (Michaud, 2004; Hall and Nguyen, 2010). Other sustainable control strategies are needed in order to reduce dependence on pesticides. Entomopathogenic fungi represent an important group of biological control agents that could be included within integrated pest management strategies.

Different *Hirsutella* species, including *Hirsutella citriformis* Speare, have been reported previously infecting a large diversity of insects (Rombach and Roberts, 1989). Population-regulating epizootics of *H. citriformis* have been reported in ACP populations in Florida (Hall et al., 2012) and in Mexico (Casique-Valdes et al., 2011; NOC, personal observation), and its pathogenicity has been evaluated in the laboratory (Meyer et al., 2007). Furthermore, the fungus *Isaria fumosorosea* Wise has been isolated and studied in ACP, confirming the potential of fungi as control agents (Meyer et al., 2008; Hoy et al., 2010; Avery et al., 2011; Stauderman et al., 2012). Although there are no reports of *Beauveria bassiana* or *Metarhizium anisopliae* infecting *D. citri*, we considered it important to include them because they are ubiquitous and abundant in the environment (Meyling and Eilenberg, 2007), and one of the *B. bassiana* isolates evaluated here was obtained from a field-infected *D. citri*. In order to select the most appropriate isolate for field trials, from many that are potentially virulent to ACP, information on other traits relevant to their potential field efficacy are required. Among the most important factors affecting fungal efficacy is temperature, particularly its effect on germination (Bouamama et al., 2010; Rivas et al., 2014), vegetative growth (Guzmán-Franco et al., 2008; Rivas et al., 2014) and infection (Fargues and Luz, 2000; Tanada and Kaya, 1993). Understanding the effect of abiotic factors, where temperature is among the most important, on selected biological attributes can provide the information necessary to predict which isolates will have the greatest potential under field conditions with specific climatic conditions. Here we describe *in vitro* experiments to elucidate the effect of temperature on growth, germination and sporulation of 16 isolates from the species *H. citriformis*, *I. fumosorosea*, *B. bassiana* s.l. Bals. Vuill. and *M. anisopliae* s.l. (Metch.) Sorokin.

2. Material and methods

2.1. Isolates

Sixteen isolates of the species *H. citriformis, B. bassiana* s.l., *I. fumosorosea* and *M. anisopliae* s.l. were used (Table 1). Some of the isolates were obtained from field-infected ACP collected in Mexico. For each isolate, a monosporic culture was produced by randomly selecting one colony-forming unit from a plate of Sabouraud dextrose agar (SDA: BIOXON®, Becton Dickinson de Mexico S.A. de C.V. Cuautitlan Izcalli, Mexico) that had been inoculated with 100 μL of a 1×10^6 conidia mL^{-1} suspension of the original isolation. All isolates (original and monosporic) were stored at -80 °C in 2 mL cryovials (Nalgene®, Thermo Fisher Scientific, Rochester, NY, USA) containing 10% sterile glycerol and deposited in the isolate collection of the Insect Pathology Laboratory, Colegio de Postgraduados, Mexico.

For experiments, conidia were harvested from subcultures of each isolate, except *H. citriformis*, grown on SDA and incubated in darkness at 25 °C for 15 days. For the *H. citriformis* isolates, the SDA was supplemented with 3% yeast extract (SDAY) and the cultures were incubated in darkness at 25 °C for 40 days. Prior to experimentation, isolates had been subcultured no more than three times after retrieval from storage at −80 °C.

Table 1
Host, geographical origin and collection date of the isolates used in this study. All locations are in México.

Species	Isolate	Host	Collection date	Geographical origin
B. bassiana s.l.	B1	*Diaphorina citri*	May, 2011	Veracruz
	B2	Cicadelidae	October, 2011	Campeche
	B3	*Bactericera cockerelli*	September, 2010	Morelos
I.		*fumosorosea*	I1	*Bemisia tabaci*
		Unknown	Yucatán	
	I2	*D. citri*	May, 2011	Veracruz
I3	*D. citri*	October, 2011	Campeche	
M.		*anisopliae* s.l.	M1	Unidentified white grub
		Unknown	Estado de México	
M2		*Aeneolamia albofasciata*	Unknown	Veracruz
H. citriformis	H1	Unidentified parasitoid	October, 2011	Campeche
	H2	“”	October, 2011	Yucatán
	H3	*D. citri*	June, 2011	Quintana Roo
	H4	“”	October, 2011	Yucatán
	H5	“”	October, 2011	Campeche
	H6	“”	October, 2011	Campeche
	H7	“”	June, 2011	Yucatán
	H8	“”	October, 2011	Campeche

2.2. Effect of temperature on in vitro growth

The effect of temperature on the growth of each isolate was assessed by measuring the area of growth that colonies achieved when incubated at 20, 25, 30 and 35 °C. The experimental procedure for *I. fumosorosea*, *B. bassiana* s.l. and *M. anisopliae* s.l. was the same, and will be described first. Conidia were harvested from cultures produced as described above and transferred to a sterile 50 mL centrifuge tube (NEPTUNE™, Continental Lab Products Inc. San Diego Cal., USA) containing 5 mL of 0.03% v/v Tween 80 and vortexed for five minutes to encourage conidia into suspension. The suspension was filtered through a sterile cloth and the concentration of conidia estimated using a haemocytometer and adjusted to 1×10^7 conidia mL^{-1}. Sterile SDA plates were each inoculated with 100 μl of this suspension and maintained at 25 °C. After 48 h, a 5 mm diameter plug was taken from the growing edge, inverted, and placed in the center of a Petri dish containing 15 mL of solidified SDA. Each dish was sealed with Parafilm ‘‘M’’ (Pechiney Plastic Packaging Inc. Wisconsin, WI, USA) and incubated at the selected temperature. Colony size was measured by creating a digitised image of each colony that was then processed using GIMP ver. 2.8 and the area of growth quantified using Image Tool ver. 3.0 software. Colony size was measured every 24 h for 15 days (if the colony had reached the edge of the plate within this time, no measurement was done after that). Four replicates of each isolate and each temperature were prepared and the whole experiment was done on two occasions (eight replicates per isolate/temperature combination overall). The temperature within each incubator was randomly allocated on each occasion and the position of the Petri dishes randomly allocated among the four shelves within each incubator.

For the *H. citriformis* isolates the method was adapted because they had slower *in vitro* growth. These isolates were first grown in SDAY broth supplemented with 3% yeast extract (SDBY); four 5 mm diameter plugs cut from the growing edge of a 40 day old colony (produced as described previously), were inoculated into 250 mL Erlenmeyer flasks each containing 50 mL of SDBY and incubated on a shaker (two flasks per isolate), at 250 r.p.m. and 25 °C for 12 days. Then, 1 mL aliquots were transferred to SDAY plates and incubated at 25 °C in darkness. After 12 days, a 5 mm diameter plug was taken from the growing edge, inverted, and placed in the center of a Petri dish containing 15 mL of solidified SDAY. Thereafter, all aspects of the experimental setup were as described above for the other fungal species, except that the colony size was measured every 48 h for 40 days.

Due to the differences in incubation time necessary to detect differences in colony size, between Group 1 species (I. fumosorosea, *B. bassiana* s.l. and *M. anisopliae* s.l. with 15 days incubation) and the *H. citriformis* isolates of Group 2 (with 40 days incubation), the sampling times for both groups had to differ substantially. Therefore data from the two groups were analyzed separately but using the same procedure. The colony area measurements over time were analyzed using a longitudinal analysis (Fitzmaurice et al., 2011), with the first-order autoregressive and compound symmetric covariance structures for isolates in Groups 1 and 2 respectively, using PROC MIXED. For isolates in both groups, we investigated whether the changes in the growth profiles over 15 days (40 days for *H. citriformis*) differed among isolates and finally we determined whether there was a significant interaction with incubation temperature (isolate × temperature). The statistical package SAS ver. 9.2 (SAS Institute, 2010) was used.

2.3. Effect of temperature on in vitro conidial germination

The germination rate was evaluated at 20, 25, 30 and 35 °C. For this experiment, three 10-μL aliquots of a 1×10^5 conidia mL^{-1} suspension of each isolate were deposited, equidistant from each other with at least two cm separating them, on to SDA (15 mL) in a 90 mm diameter Petri dish. For each isolate (except *H. citriformis* isolates), 18 SDA plates were prepared as described above for each temperature to be evaluated; three replicate plates were destructively sampled after 4, 8, 12, 16, 20 and 24 h of incubation and the germination of conidia assessed. For the *H. citriformis* isolates, the experimental setup was as described above except that 24 plates were prepared for each temperature to be evaluated, and three replicate plates were destructively sampled and germination evaluated after 24, 48, 72, 96, 120, 144, 168 and 192 h of incubation. To assess germination after each incubation time, conidia were fixed with 10% cotton blue in lactophenol. Using a microscope, the proportion of germinated conidia out of 100 conidia randomly selected, was quantified in each of the 10 μL aliquots of conidial suspension deposited per plate. Conidia were considered as germinated if the germ tube was longer than the diameter of the conidium.

The experiment was done using a completely randomized design with three replicates per treatments and the complete experiment was repeated on two different occasions (six replicates in total). Data were analyzed using logistic regression (assumed to follow a binomial distribution) and a sample size equal to the number of conidia evaluated. Each value of germination was a proportion of the total number of conidia evaluated. Data from the four hours estimation was excluded from the analysis, as no conidia had germinated at this sampling time. Data from the *H. citriformis* isolates were analyzed similarly, but separately from the other isolates. Analysis for this experiment was done using the statistical package GenStat v 8.0 (Payne et al., 2005).

2.4. Effect of temperature on in vitro sporulation

The number of conidia produced per isolate on 90 mm diameter SDA plates was estimated. For each isolate, replicate 90 mm diameter Petri dishes containing 15 mL of SDA (SDAY for *H. citriformis* isolates) were prepared and each one inoculated by spreading 500 μL of a 1×10^7 conidia mL^{-1} concentration suspension. Four replicate Petri dishes were incubated for each isolate at each temperature: 20, 25, 30, 35 °C. After 15 days Petri dishes from each isolate × temperature combination of *I. fumosorosea*, *B. bassiana* s.l. and *M. anisopliae* s.l., were removed from incubators. Although it would have been desirable to estimate the conidia production from the complete colony, due to the large number of conidia produced by these fungal isolates, conidia production was estimated from a subsample of each colony. For this, three 8 mm diameter plugs were taken from each Petri dish; as the fungal inoculum had been spread over the surface, this allowed us to randomly select positions from which to remove plugs in each Petri dish. Each plug was deposited individually into a 50 mL centrifuge tube containing 5 mL of 0.03% Tween 80. Centrifuge tubes were vortexed for two minutes to dislodge conidia. For the *H. citriformis* isolates, plates were incubated for 40 days rather than 15 days. Because this fungal species produced fewer conidia compared to the other fungal species, it was possible to estimate the conidia production from the entire colony; therefore, conidia were harvested from the complete surface of the Petri dish using a sterile scalpel and vortexed for 10 min rather than two minutes. Conidial suspensions from all isolates were filtered through a sterile muslin cloth (to remove large hyphae and medium residues), into a new sterile centrifuge tube. A sample of the resulting suspension was diluted 100 fold (10 μL of the conidial suspension in 990 μL of 0.03% Tween 80 solution) and the conidial concentration estimated using a haemocytometer. The experimental design, data handling and statistical analysis were the same as described previously for the germination experiment. No sporulation was obtained with isolate H4 at any of the temperatures, and at 35 °C for all isolates, therefore data from these treatments were excluded from the analysis. Analysis for this experiment was done using the statistical package GenStat v 8.0 (Payne et al., 2005).

3. Results

3.1. Effect of temperature on in vitro growth

Significant differences in *in vitro* growth were found among Group 1 isolates ($F_{7,512} = 935.84$, $P < 0.0001$), where the greatest growth was achieved by the *M. anisopliae* isolate M2 (Fig. 1). Overall, greatest growth was achieved at 25 and 30 °C, followed by 20 °C and 35 °C (Fig. 1d) ($F_{3,512} = 3614.23$, $P < 0.001$). There was a significant interaction between isolate and temperature ($F_{21,512} = 135.17$, $P < 0.001$); despite the fact that the growth of isolate M2 was always greater than the other isolates, the difference was more evident at 25 and 30 °C (Fig. 1b and c).

Significant differences in *in vitro* growth were also found among isolates of *H. citriformis* ($F_{7,640} = 1135.63$, $P < 0.001$). Overall, the greatest *in vitro* growth was achieved by isolate H2 (Fig. 2). Overall, the greatest growth was achieved at 25 °C, followed by 20, and 30 °C, with no growth at 35 °C (Fig. 2d) ($F_{3,640} = 34998.7$, $P < 0.001$). There was a significant interaction between isolate and temperature ($F_{21,640} = 380.25$, $P < 0.001$), where isolate H2 showed the greatest growth at 25 °C, followed by isolates H1 and H6 (Fig. 2b).

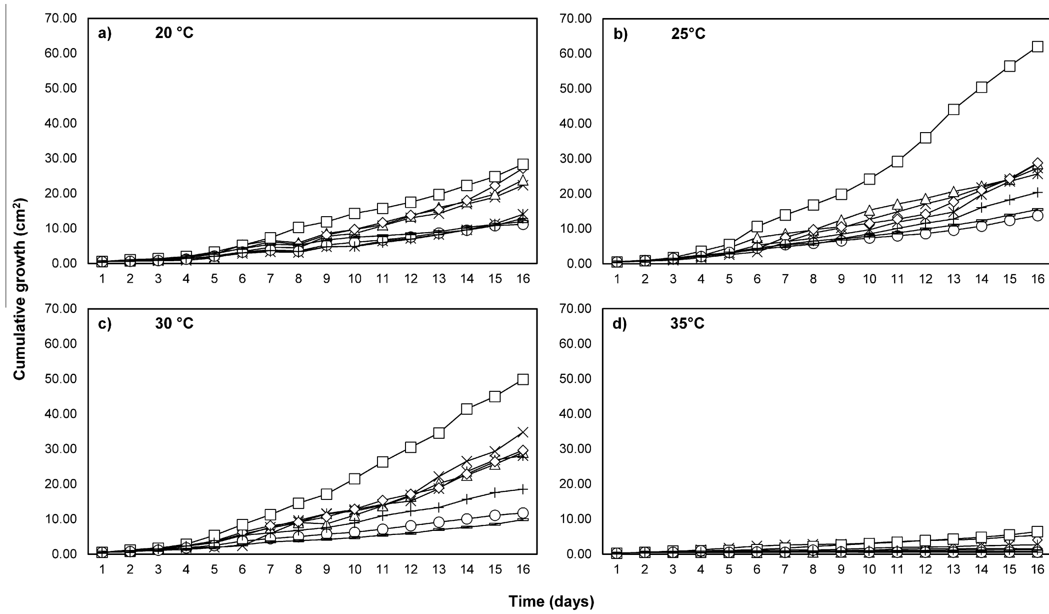

Fig. 1. Cumulative growth of the Group 1 isolates at four different temperatures (a) 20 °C, (b) 25 °C, (c) 30 °C, (d) 35 °C. B1 (), B2 (), B3, (), M1 (), M2 (), I1 (), I2 (), I3 (). More information on the isolates is presented in Table 1.

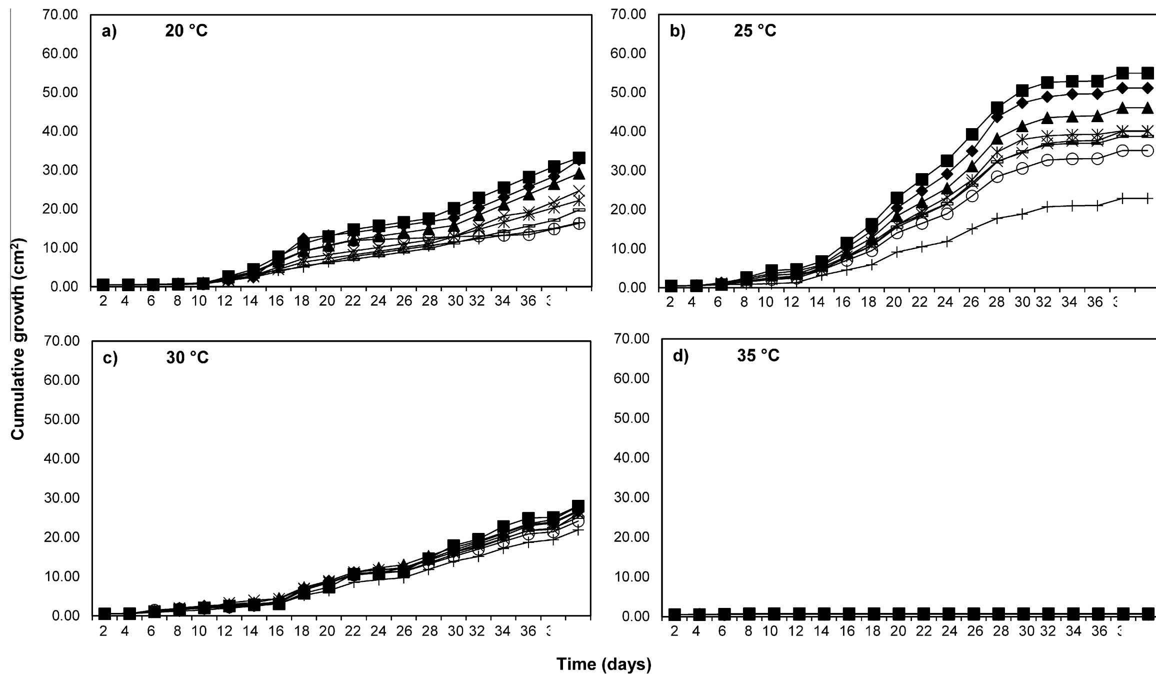

Fig. 2. Cumulative growth of Group 2 (*H. citriformis*) isolates at (a) 20 °C, (b) 25 °C, (c) 30 °C, (d) 35 °C. H1 (), H2 (), H3 (), H4 (), H5 (), H6 (), H7 (), H8 (). More information on the isolates is presented in Table 1.

3.2. Effect of temperature on in vitro conidial germination

Germination rates were greatly affected by isolate ($F_{7,800}$ = 229.60, P < 0.001), sampling time ($F_{4,800}$ = 4280.07, P < 0.001) and temperature ($F_{3,800}$ = 8930.41, P < 0.001), with a significant interaction among these factors ($F_{84,800}$ = 3.23, P < 0.001). Some germination was observed within eight hours of setting up the experiments (Fig. 3) and nearly 100% germination was achieved after 16 h of incubation (Fig. 3a–d). The *M. anisopliae* isolates M1 and M2 achieved the greatest germination rate after 8 h of incubation at 25 and 30 °C (Fig. 3b and c). Germination was greatly reduced at 35 °C for all isolates and all incubation times, although isolates M1, M2 and I2 showed significant germination after 16 h of incubation (Fig. 3d).

Germination of conidia from *H. citriformis* was significantly different among isolates ($F_{7,1120}$ = 1514.57, P < 0.001), sampling time

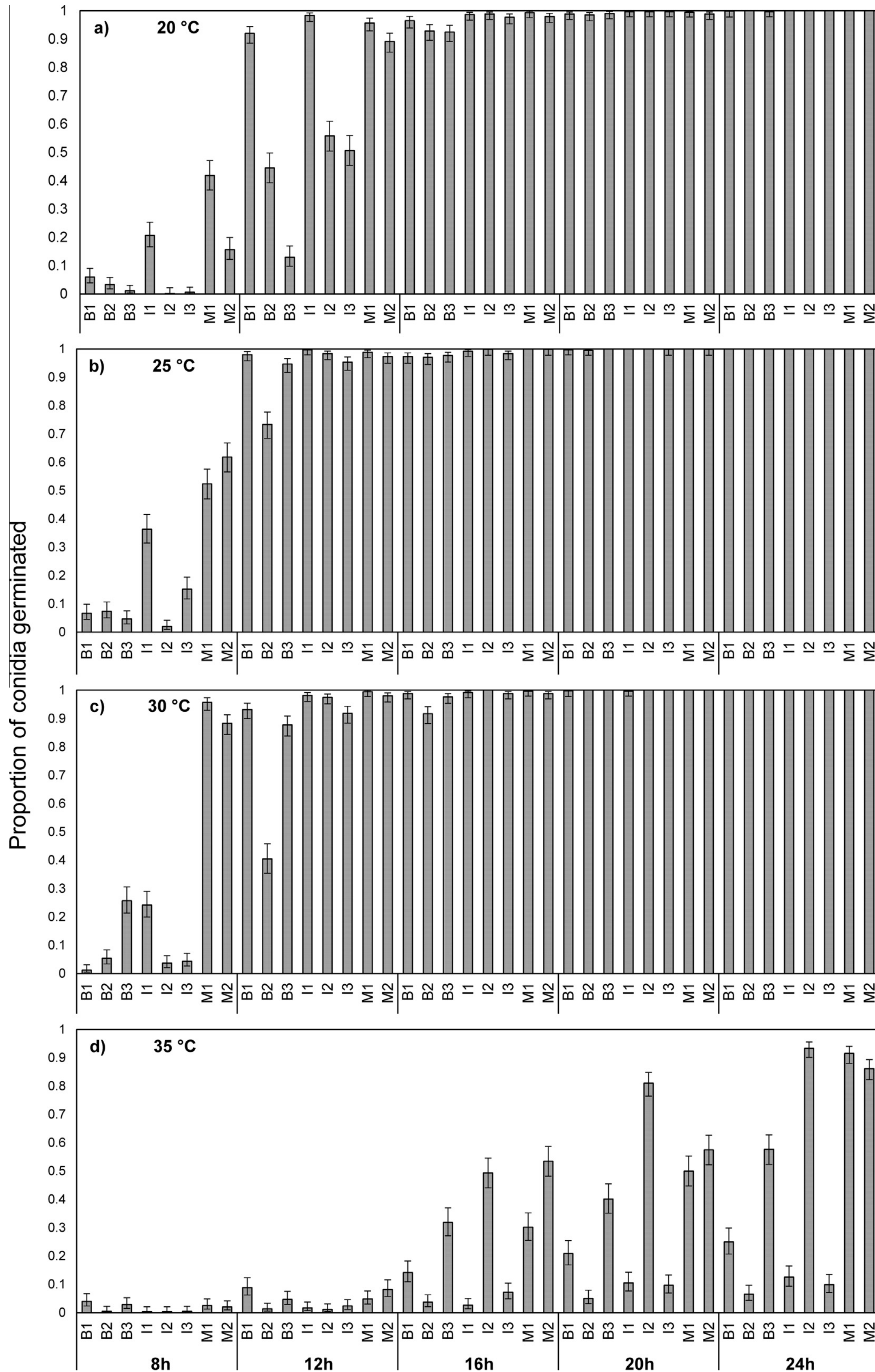

Fig. 3. *In vitro* germination of Group 1 isolates after six different incubation times at (a) 20 °C, (b) 25 °C, (c) 30 °C, (d) 35 °C. Error bars represent 95% confidence intervals back-transformed from the logistic scale.

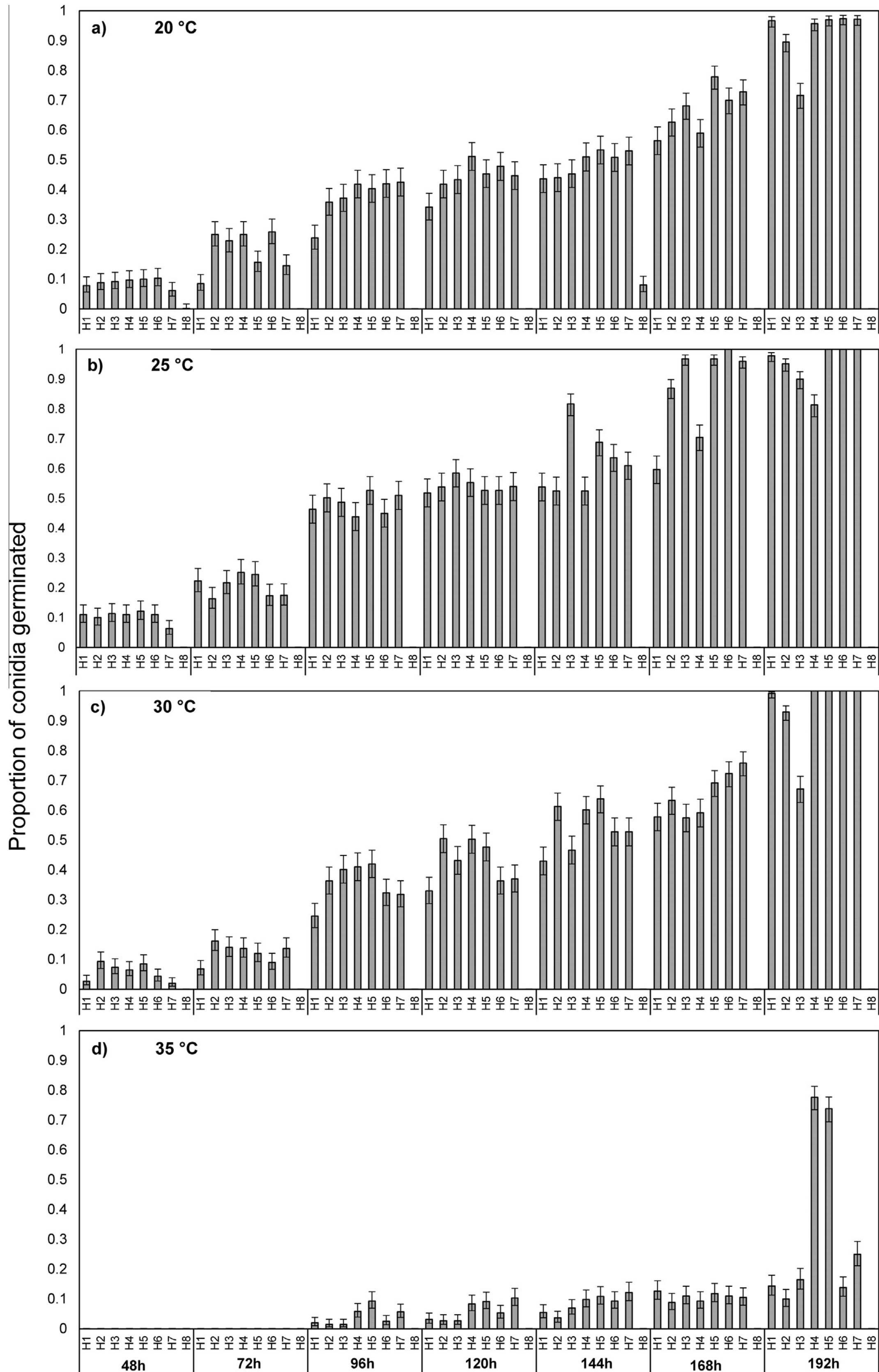

Fig. 4. *In vitro* germination of Group 2 (*H. citriformis*) isolates after six different incubation times at (a) 20 °C, (b) 25 °C, (c) 30 °C, (d) 35 °C. Error bars represent 95% confidence intervals back-transformed from the logistic scale.

($F_{6,1120}$ = 3513.85, P < 0.001) and temperature ($F_{3,1120}$ = 5509.30, P < 0.001), with a significant interaction among them ($F_{126,1120}$ = 10.03, P < 0.001). Overall, from 20 to 30 °C, germination of the majority of the isolates (except H8) was greater than 40% after 96 h incubation (Fig. 4a–c), with a maximum of 10% for only one isolate (H5) at 35 °C after the same time interval (Fig. 4d). Isolates H4 to H7 reached 100% germination at 30 °C after 192 h of inoculation (Fig. 4c), and nearly 100% at 20 and 25 °C after the same time interval (Fig. 4a and b). Only isolates H4 and H5 achieved approximately 80% germination at 35 °C after 192 h of inoculation (Fig. 4d). Isolate H8 never germinated at any temperatures or time intervals, except at 20 and 25 °C after 144 h of inoculation (approximately 10%) (Fig. 4a and b).

3.3. Effect of temperature on in vitro sporulation

There were significant differences in *in vitro* sporulation among isolates ($F_{7,83}$ = 148.14, P < 0.001), where the greatest sporulation was achieved by *B. bassiana* isolate B1 (Fig. 5a). There was also a significant interaction with temperature ($F_{18,83}$ = 99.69, P < 0.001). Isolate B1 showed the greatest sporulation at 20 °C followed by isolate I1 (Fig. 5a), but at 25 °C, isolates B1 and B2 showed similar sporulation, which was greater than the other isolates (Fig. 5b and c). Overall, the lowest sporulation was achieved at 35 °C for all isolates, especially for the *B. bassiana* isolates (Fig. 5d).

There were significant differences in sporulation among *H. citriformis* isolates ($F_{6,60}$ = 37.55, P < 0.001) and there was a significant interaction with temperature ($F_{12,60}$ = 100.01, P < 0.001); isolates H2 and H8 had the greatest sporulation but only at 25 °C (Fig. 6b), and isolates H1 (Fig. 6a) and H7 (Fig. 6c) had the greatest sporulation at 20 and 30 °C respectively. Overall, isolate H2 showed the greatest sporulation, followed by isolate H7 (Fig. 6).

4. Discussion

Abiotic factors such as light, humidity and temperature affect the stability and persistence of entomopathogenic fungi (Fargues et al., 1997; Fargues and Luz, 1998). Specifically, temperature affects germination and growth (Tefera and Pringle, 2003; Uma et al., 2005). The responses of fungi to different temperatures have been extensively studied, and inter and intraspecific differential responses have been reported. This information has been used to select potential isolates to be developed as biological control agents (Orduño-Cruz et al., 2011; Petlamul and Prasertsan, 2012; Ibarra-Cortés et al., 2013).

Our results showed significant differences in the *in vitro* growth of isolates of *B. bassiana*, *M. anisopliae* and *I. fumosorosea*. Overall, *M. anisopliae* and *B. bassiana* isolates showed greater growth at 25 and 30 °C compared to the other temperatures tested, whereas *I. fumosorosea* showed significantly more growth only at 25 °C. Our findings are in line with previous reports for *B. bassiana* and *M. anisopliae* (Fargues et al., 1997; Ouedraogo et al., 1997; Orduño-Cruz et al., 2011; Ibarra-Cortés et al., 2013). At 35 °C, only isolates B1 (*B. bassiana*), M1 and M2 (*M. anisopliae*) showed any growth (approx. 2.5 cm^2) compared with the other isolates where no growth was observed. The intraspecific variation among isolates growing above 30 °C has been related to their geographical origin. For example, the isolates we tested that grew at 35 °C were isolated from the states of Veracruz and Yucatan in Mexico, where the average temperatures (including day and night temperatures) are 32 and 36 °C respectively. The relationship between optimal temperature for *in vitro* growth and geographical origin has been reported previously for *B. bassiana, Beauveria brongniartii, Metarhizium flavoviride, Nomuraea rileyi* (Farlow) Samson and *I. fumosorosea* (Fargues et al., 1992; Vidal et al., 1997). We hypothesize that the isolates that showed the greatest values in all biological attributes assessed at the majority of temperatures evaluated, will have a greater capacity for adaptation to the fluctuating temperatures present in Veracruz and Yucatan, where *D. citri* is a serious problem on citrus.

Conidial germination is one of the most important steps during the infection process of entomopathogenic fungi, and can be greatly affected by temperature (Talaei-Hassanloui et al., 2007). Our results showed that germination rate was indeed modified by temperature, at least 16 h post inoculation. Greatest germination occurred at 25 and 30 °C, rather than 35 °C, where germination was greatly delayed for all isolates, especially for *M. anisopliae*. Previous reports have stated that the optimal temperature for germination of both *M. anisopliae* (Tanada and Kaya, 1993) and *B. bassiana* (Ekesi et al., 1999) is 25 °C so our results are in line with these. However, we observed that the rate of germination also varied among fungal species; for example, all *M. anisopliae* isolates achieved practically 100% germination within16 h, whereas isolates of *B. bassiana* and *I. fumosorosea* took 20 h at all temperatures except 35 °C.

Conidia production, either *in vivo* or *in vitro*, is an important factor that should be considered when selecting isolates (Posada and Vega, 2005). However, the use of blastospores of *I. fumosorosea* for the control of *D. citri* (Avery et al., 2011; Stauderman et al., 2012), has shown promising results. Blastospores of *I. fumosorosea* has been commercialized in Europe with the trade name of Preferal® (Faria and Wraight, 2007). Furthermore, its compatibility with fungicides commonly used in citrus orchards was also studied, emphasizing its potential for inclusion in integrated pest management programmes for *D. citri*, and warranting further development (Avery et al., 2013). A greater capacity for sporulation increases the chances of the fungus infecting more hosts and potentially establishing in the field. Our results showed an overall negative relationship between temperature and *in vitro* production of conidia because the higher the incubation temperature was, the smaller the number of conidia that were produced. The largest number of conidia were produced between 25 and 30 °C, and some sporulation occurred at 35 °C; only one isolate of *B. bassiana,* two of *M. anisopliae* and two of *I. fumosorosea* were able to produce some conidia at 35 °C. Variation in conidia production can be related to specific biological attributes of isolates or the artificial medium on which they are grown (Petlamul and Prasertsan, 2012). Temperature and humidity have also been suggested as major factors affecting sporulation (Arthurs and Thomas, 2001; Tefera and Pringle, 2003).

To our knowledge, our results with *H. citriformis* isolates represent the first report of the effect of temperature on their *in vitro* growth, germination and sporulation. Overall, *H. citriformis* isolates had the slowest growth, germination and sporulation of all the fungal species evaluated. For example, only 30% of conidia had germinated after 96 h and only 60% germination was achieved after 168 h incubation at temperatures between 20 and 30 °C. *H. citriformis* conidia are covered with preformed mucus which helps them disperse and avoid desiccation. However, we hypothesize that when conidia were suspended in 0.03% Tween 80, which is known to be a surfactant, that the mucus was removed having a negative effect on germination; we have tested this experimentally and results will be presented in a separate paper. There is no published information regarding the properties of mucus and its relationship with germination and infection, and so this requires further study. Additionally, the number of conidia produced by *H. citriformis* isolates was smaller compared to the other fungal isolates, which may be as a result of its biology; only one conidium is produced per phialide (Meyer et al., 2007).

From these studies, isolates of *I. fumosorosea*, *B. bassiana* s.l. and *M. anisopliae* s.l. had the greatest potential to be developed as

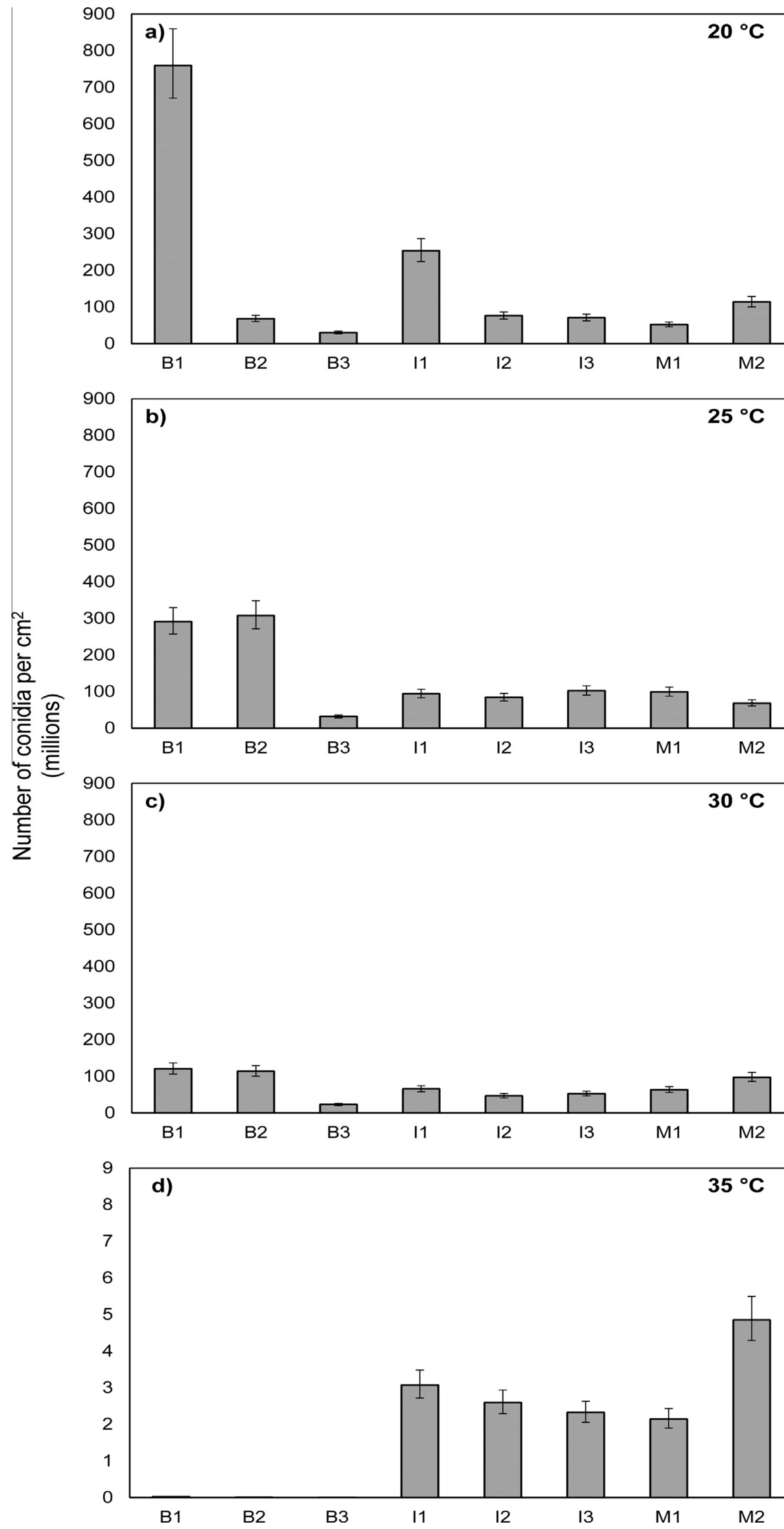

Fig. 5. *In vitro* sporulation of Group 1 isolates at (a) 20 °C, (b) 25 °C, (c) 30 °C, (d) 35 °C. Error bars represent 95% confidence intervals back-transformed from the logarithmic scale.

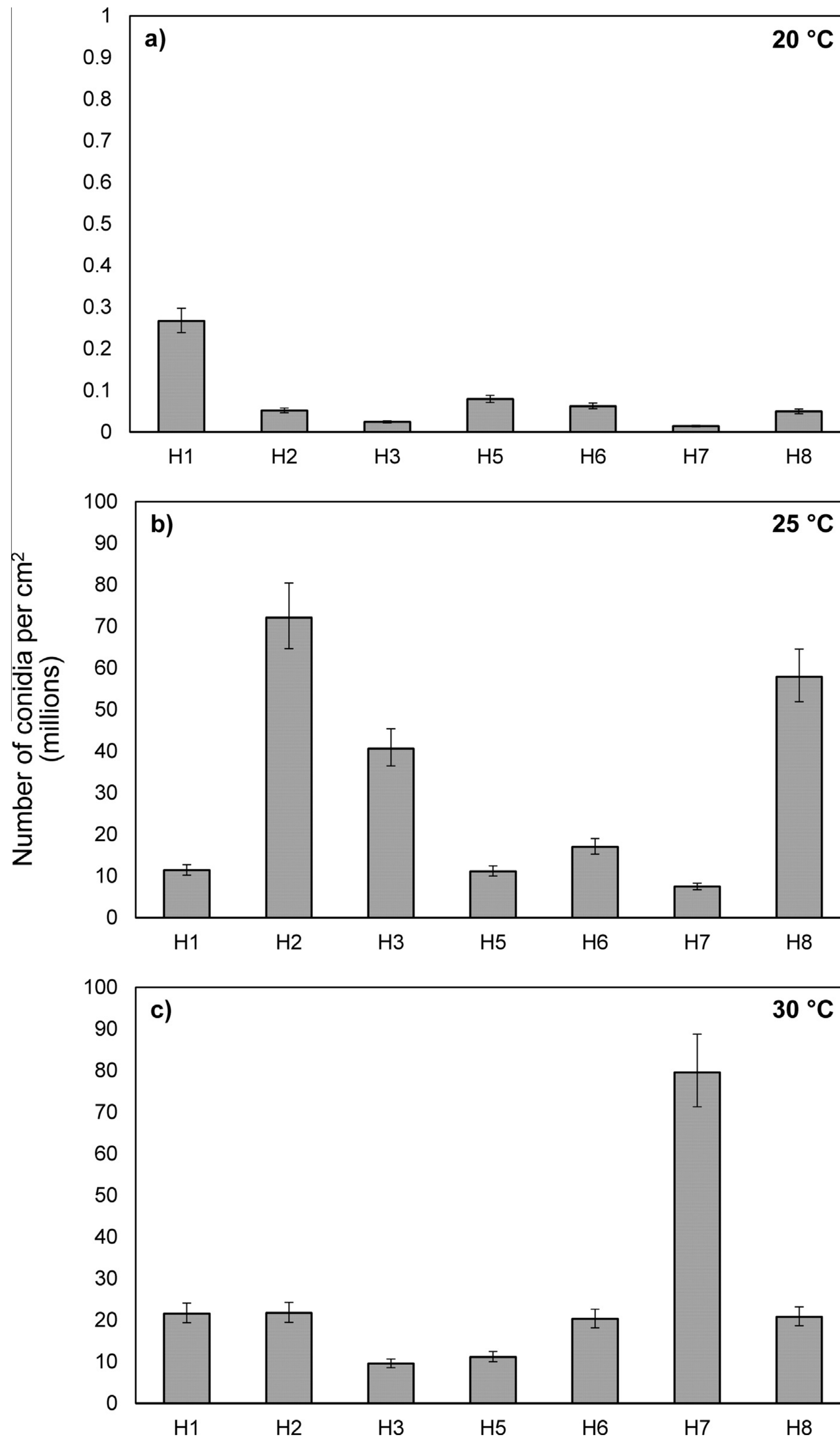

Fig. 6. *In vitro* sporulation of Group 2 (*H. citriformis*) isolates at (a) 20 °C, (b) 25 °C, (c) 30 °C. No sporulation was obtained at 35 °C. Error bars represent 95% confidence intervals back-transformed from the logarithmic scale.

biological control agents. They also had the advantage that they can be sprayed conventionally as aqueous conidial suspensions. However, we cannot rule out the use of *H. citriformis* for the control of *D. citri*, as this species is the one found most commonly infecting natural populations of this insect in the field (Hall et al., 2012; NOC, personal observation). The regular presence of *H. citriformis* in *D. citri* populations in nature suggests a close co-evolution between the two species. If this relationship can be better understood it may be possible to exploit *H. citriformis* more successfully using other strategies, such as conservation biological control approaches.

In conclusion, isolates B1, M2 and I1 outcompeted all other isolates with respect to the biological attributes evaluated here, suggesting they have greatest potential to be developed as biological

control agents for control of *D. citri*. The *in vivo* interactions between these isolates and *D. citri* are currently under investigation. In addition, *in vivo* experiments will also be done for *H. citriformis* isolates. The host-pathogen interaction is complex, and a combination of *in vivo* and *in vitro* data will provide a better base for the selection of isolates for field evaluation.

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