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Phenotype responses to abiotic stresses, asexual reproduction and virulence among isolates of the entomopathogenic fungus *Cordyceps javanica* (Hypocreales: Cordycipitaceae)

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ABSTRACT

Selecting entomopathogenic fungal isolates with resilience to environmental stresses, optimal mass production characteristics, and with high virulence to target pests favors the development of mycopesticides. A case in point, *Cordyceps* (= *Isaria*) *javanica* has been extensively investigated for non-chemical control of whiteflies worldwide. We phylogenetically characterized 11 native *C. javanica* isolates from Northeastern and Central Brazil. These isolates were screened for tolerance to heat-shock, UV-B radiation, osmotic and oxidative stresses, as well as conidial production on cereal grain and insecticidal activity against the whitefly *Bemisia tabaci* (MEAM 1) in the laboratory. All isolates were pathogenic to whiteflies and significant (3-fold) differences in median lethal concentration were observed among isolates. Furthermore, pronounced differences among isolates were found for stress factors and conidial production. Using principal component analysis, our results highlighted three major clusters formed by isolates (i) resistant to osmotic and oxidative stress, (ii) resilient to UV-B, and (iii) with high virulence, conidial production and heat tolerance. Overall, isolate CG1228 performed best based on multi-stress resistance, mass production and virulence attributes in the laboratory. This study highlights the importance of exploring natural variation in entomopathogenic fungi for selection of appropriate isolates for effective biocontrol of insect pests coupled with mass production characteristics and abiotic stress tolerances.

1. Introduction

Fungal entomopathogens are ubiquitous in nature and several, i.e. among the Hypocreales, have been developed as mycoinsecticides and mycoacaracides (Faria and Wraight, 2007; Samson et al., 2013; Lacey et al., 2015). Despite this, the susceptibility of fungal propagules (conidia and blastospores) to ultraviolet (UV-B) radiation and other deleterious environmental factors limit the practical use of mycopesticides in many cases (Inglis et al., 1997; Braga et al., 2001; Fernández-Bravo et al., 2016). However, differences in environmental tolerances occur among isolates of entomopathogenic fungi due to their phenotypic plasticity (Ignoffo and Garcia, 1992; Rangel et al., 2005; Fernandes et al., 2007, 2015). Moreover, recent studies have documented 'stress phenotypes' of entomopathogenic fungi which can be induced through controlling physical, chemical and nutritional conditions during *in vitro* mycelial growth (Rangel et al., 2015). It is clear that selecting or manipulating fungal isolates for environmental competence is a major factor determining their effectiveness under field conditions (Jackson and O'Callahan, 1997).

In Brazil, the entomopathogenic fungi *Metarhizium anisopliae* (Metsch.) Sorok. sensu lato (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Bals.-Criv.) Vuill. sensu lato (Hypocreales: Cordycipitaceae) have been extensively used to control several major agricultural and forest insect pests since the 1970s (Mascarin et al., 2018). More recently, the *Isaria* species complex [proposed new name *Cordyceps* (Kepler et al., 2017)] has been researched for the control of whiteflies, particularly the silverleaf whitefly *Bemisia tabaci* Genn. (Hemiptera: Aleyrodidae) Middle East Asia Minor 1 (MEAM 1)

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(formerly biotype B) (Mascarin et al., 2013). MEAM 1 is an economically significant pest in Brazil, and infests crops including soybean, cotton, bean, melon, tomato and more recently corn where it vectors several plant-pathogenic geminiviruses (Inoue-Nagata et al., 2016; Quintela et al., 2016). In some regions, persistent insecticide campaigns against MEAN 1 has led to the development of resistance (Basit et al., 2013; Horowitz and Ishaaya, 2014).

Mycoinsecticides can provide tools for insecticide resistance management and organic control methods for MEAM 1. In Europe and North America, commercial blastospore formulations of *Cordyceps fumosorosea* [formerly *Isaria fumosorosea* (Kepler et al., 2017)] (Wize) Kepler, B. Shrestha & Spatafora, comb. nov. (Hypocreales: Cordycipitaceae) are sold for the control of whiteflies and other soft-bodied insects (Zimmermann, 2008; Avery et al., 2013). We previously demonstrated that *Cordyceps javanica* blastospores and conidia infective to whiteflies can be cultured in large quantities using low-cost *in vitro* approaches (Mascarin et al., 2015). We also identified *C. javanica* isolate CG1228 as a possible candidate for whitefly biocontrol due to its notable mass production characteristics on solid substrate and virulence against MEAM 1 life stages (Mascarin et al., 2013).

Efforts to screen native *Cordyceps* (= *Isaria*) isolates with high virulence and mass production characteristics, along with improved tolerances to detrimental environmental factors, such as UV radiation, elevated temperatures, oxidative and osmotic (low water availability) stress, is warranted to select candidates for development as robust mycoinsecticides. Phenotype selection within entomopathogenic fungi is scarce, yet can improve fungal isolates without resorting to transgenic constructs. As a result, selected entomopathogenic fungal isolates holding desirable traits in stress tolerance, production and virulence enables the development of selection programs for improved isolates. Here, we investigated correlations of virulence, multi-stress tolerances and conidial production among several *C. javanica* isolates collected in Brazil. Our results are used to conduct multivariate analysis using phenotypic traits to identify the most promising isolates for development as a mycoinsecticide. Natural variation among fungal isolates also offers an approach to investigate molecular and genetic mechanisms underlying stress tolerance, virulence and asexual reproduction tradeoffs in an evolutionary context.

2. Materials and methods

2.1. Fungal collections

The *C. javanica* isolates were obtained from symptomatic mycosed *B. tabaci* MEAN 1 nymphs or adults collected from Northeastern and Central Brazil over 5 years (Table 1). An exception was CG1228 which originated from a mycosed larva of *Rupela albinella* (Cramer) (Lepidoptera: Crambidae). Dead insects were surface-sterilized in 70% ethanol and 1% hypochlorite for 30 s, and then rinsed twice in sterile deionized water before plating on water-agar (2% w/v) medium. After 4–6 days incubation (26 ± 0.5 °C and 12 h photoperiod, Philips® 20 W, cool fluorescent lamps), aerial conidia from cadavers were transferred to potato-dextrose-agar (PDA, Neogen Corp., Lansing, MI, USA) using a sterile loop. Conidia from purified PDA cultures were used for bioassays and molecular identification after two weeks. Fungi were sub-cultured on PDA medium not more than four times before bioassays, to minimize attenuation (Ansari and Butt, 2011). All isolates were preserved in liquid nitrogen and deposited at the Invertebrate Fungal Collection at Embrapa Genetic Resources and Biotechnology (CG), Brasília-DF, Brazil.

2.2. Molecular identification of isolates

For fungal identification, polymerase chain reaction (PCR) amplifications of the partial β-tubulin gene were performed with 1x AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA), ~100 ng template DNA, and 0.2 µM each of primers bt2a and bt2b (Glass and Donaldson, 1995). The β-tubulin gene PCR reactions were performed as follows: initial denaturation of 10 min at 95 °C; 35 cycles, each consisting of a 30 s denaturation at 95 °C, a 30 s annealing at 55 °C, and a 1 min extension at 72 °C; final extension step for 7 min at 72 °C. Amplification products were purified using Montage PCR Cleanup Filter Plates (Millipore, Billerica, MA, USA). Sequencing reactions were conducted using the ABI BigDye version 3.0 sequencing kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's suggested protocol, but at one-tenth the recommended volume. Reaction products were purified using the BigDye XTerminator® Purification Kit (Applied Biosystems, Foster City, CA, USA) and sequenced on an ABI3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA) using the aforementioned oligonucleotide primers. Resulting DNA sequences were assembled using Sequencer® 5.4 software (Gene Codes Corporation, Ann Arbor, MI, USA). Consensus sequences were blasted against the EntomopathogenID database (Dunlap et al., 2017a, 2017b) and sequence data of closely related isolates downloaded. Phylogenetic analysis was performed in MEGA 7 (Kumar et al., 2016) using the Neighbor-joining (NJ) method (Saitou and Nei, 1987). For NJ analysis, the Kimura two parameter model with non-uniformity of substitution rates modeled by a gamma distribution (shape parameter = 0.3) was fit using the likelihood-based model selection algorithm in MEGA 7. The complete deletion option was used, and the level of bootstrap support was calculated from 1000 replicates.

Table 1
Molecular identification of *Cordyceps javanica* isolates collected in Brazil along with place of origin, host/crop and collection year.

Isolate[§]	GenBank accession number[†]	Geographic origin	Crop	Year of collection
CG1228[*]	MG659303	Arari, MA	Rice	2011
CG1282	MG659304	Porangatu, GO	Soybean	2013
CG1281	MG659305	Sto. Antônio de Goiás, GO	Bean	2013
CG1300	MG659306	Planaltina, GO	Corn	2013
CG1284	MG659307	Planaltina, GO	Bean	2013
CG1286	MG659308	Planaltina, GO	Guava	2013
CG1285	MG659309	Planaltina, GO	Tomato	2013
CG1283	MG659310	St. Antônio de Goiás, GO	Bean	2013
CG1315	MG659311	Brasília, DF	Bean	2015
CG1316	MG659312	St. Antônio de Goiás, GO	Cotton	2015
CG1326	MG659313	Goianira, GO	Bean	2015

[§] Culture collection: CG, Centro Nacional de Recursos Genéticos – CENARGEN, Embrapa, Brazil.

[*] This isolate was passages via whitefly nymphs and re-isolated prior to use in bioassays.

[†] Molecular identification based on the sequencing analysis of beta-tubulin gene (*TUB2*). All isolates were obtained from *Bemisia tabaci* (Genn.) MEAM1 (formerly biotype B) except CG1228, which came from *Rupela albinella* (Cramer) (Lepidoptera: Crambidae).

2.3. Heat shock stress

Conidial thermotolerance was assayed based on previous methods (Rangel et al., 2005; Fernandes et al., 2008). All isolates were cultured on PDA supplemented with yeast extract 1 g/L (Technical, Difco™ Detroit, MI, USA) (PDAY) in 9-cm Petri plates in the dark at 27 ± 1 °C for 15 days. Conidia were harvested with a sterile loop, filtered through cheesecloth, and suspended in sterile water containing 0.01% v/v Tween® 80 (Sigma Chemical Co., St Louis, MO, USA). The concentration was adjusted to 10^6 conidia/mL and 2 mL transferred to 15 × 150 mm glass screwcap tubes and placed in a 45 ± 0.1 °C water bath (model NL-21-02, New Lab, Piracicaba, SP, Brazil). Conidia were exposed for 0 (control), 1, 2, 4 or 6 h (heat stress treatment) and afterwards 20 µL of

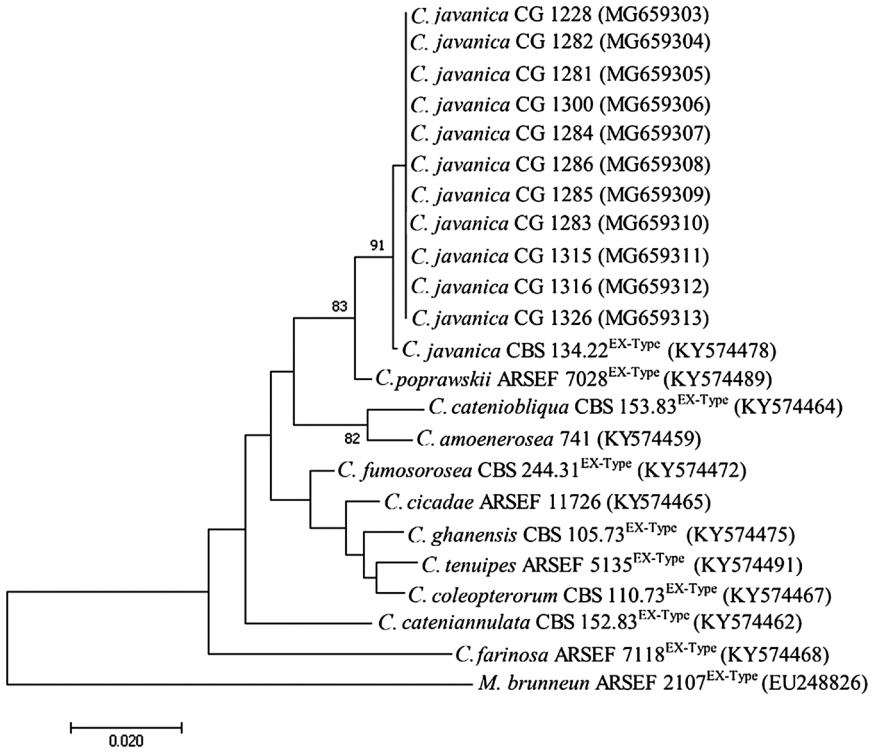

Fig. 1. Phylogenetic Neighbor-joining tree reconstructed from the partial β-tubulin gene of eleven isolates and type strains from the *Cordyceps* genus. The scale bar corresponds to 0.02 nucleotide substitutions per site and only bootstrap values greater than 80% are shown. *Metarhizium brunneum* was used as an outgroup.

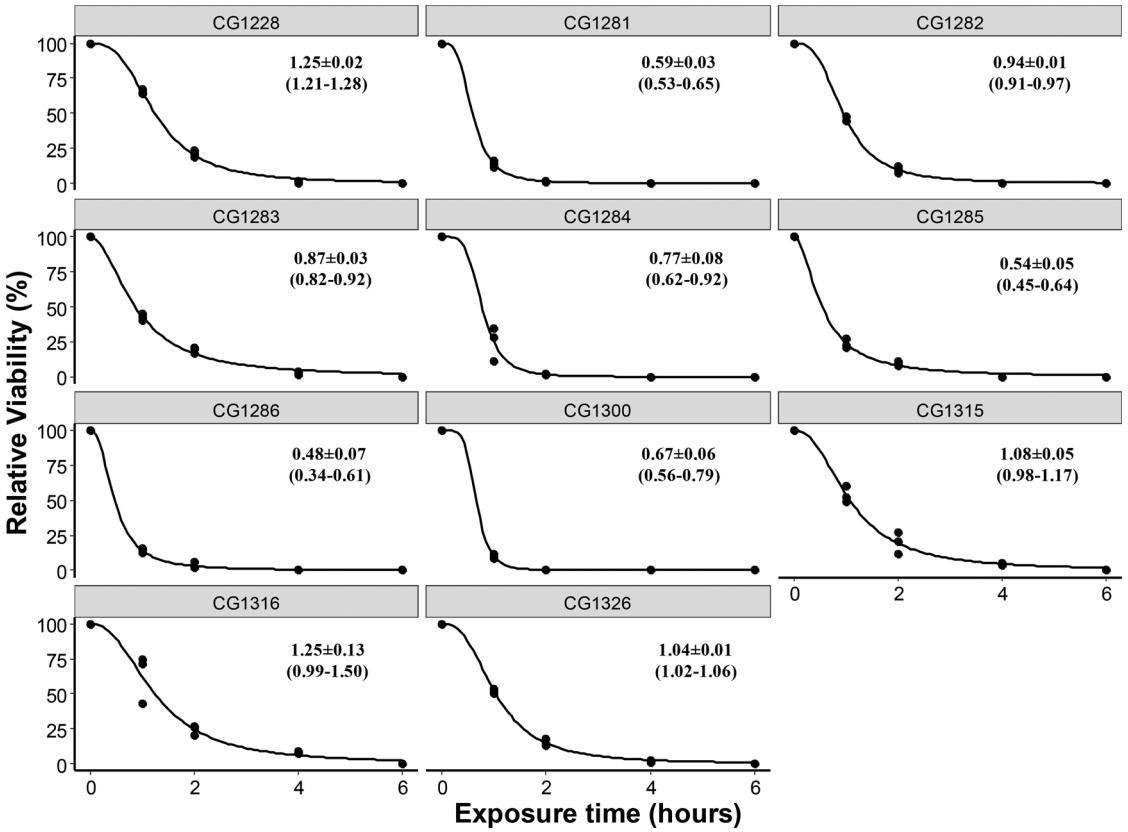

Fig. 2. Tolerance of *Cordyceps javanica* isolates to heat shock. Data points show relative conidial viability on PDA after 48 h following exposures to heat stress (45 °C) and solid lines are fitted Gompertz (clog-log) models. Values inside plots correspond to predicted median effective times (ET_{50} ± standard error) corresponding to 50% relative viability with 95% confidence intervals. The relative viability was computed in relation to the control group (i.e., without heat exposure) for each isolate.

suspension was inoculated in the center (without spreading) of a 6-cm Petri plates with PDAY medium plus 0.002% w/v benomyl (Hi-Yield Chemical Company, Bonham, TX, USA) and 0.05% w/v chloramphenicol (Officinal, Goiânia, Brazil). The benomyl was used to prevent hyphal outgrowth (Milner et al., 1991). The plates were incubated for 48 h in darkness at 27 ± 1 °C and then conidia were stained with lactophenol blue solution to assess germination. Conidia were considered germinated when germ tubes measured at least conidial diameter.

2.4. UV-B stress

Conidial UV-B resistance was assayed using the protocol of Fernandes et al. (2007). Isolates were prepared as described above. Twenty µL suspension aliquots (10^6 conidia mL^{-1}) were centrally spotted (without spreading) on 6-cm PDAY plates plus benomyl and chloramphenicol and exposed to 378 mW/m^2 of Quaite-weighted irradiance (Quaite et al., 1992) for 2, 3, or 4 h in a UV-B chamber, providing adose of 2.72, 4.08, and 5.44 kJ/m^2, respectively. The chamber was illuminated by two ultraviolet lamps (UVB-313 EL/40 W, Q-Lab Corporation, Westlake, OH, USA), and the spectral irradiance was measured by an USB 2000 + Rad Spectroradiometer (Ocean Optics, Dunedin, FL, USA) (Figure S1 in Supplementary Material). Irradiated plates, before exposure, were covered with a 0.13 mm-thick cellulose diacetate film (JCS Industries, Spring, TX, USA) which blocks UV-C radiation (below 280 nm) and short-wavelength UV-B (280–290 nm), but allows the passage of emitted UV-B (290–320 nm) and UV-A (320–400 nm). The UV-B chamber was placed in a temperature-controlled room with the temperature inside the chamber maintained at 27 ± 1 °C during tests, as measured with a HOBO® datalogger (Onset, Bourne, MA, USA). Control plates were covered with aluminum foil before placement in the chamber. Irradiated plates were incubated for 48 h at 27 ± 1 °C in the dark, and conidial viability was assessed.

2.5. Osmotic stress

This test was performed to simulate conditions experienced by fungal spores under low ambient relative humidity. Osmotic stress also constrains fungal development in the host hemolymph (Wang et al., 2008). Conidial tolerance to hyperosmotic environment (i.e., simulating germination under water stress) was assessed by adding NaCl (Sigma®, St. Louis, MO, USA) prior to PDA medium sterilization at 0 (control), 0.5, 1.0, 1.5, and 2.0 M (mol/L). A 200-µL aliquot of each fungal suspension containing 5×10^5 conidia/mL was placed on the center of each NaCl-adjusted PDA treatment. Osmotic pressure was calculated from: $\Pi = (i \times C \times R \times T)/1000$, where Π = osmotic pressure (MPa), $i = 2$ (ionic dissociation of NaCl), C = molarity of NaCl (mol L^{-1}), $R = 8.3145$ L kPa/mol/K (gas constant), $T = 299.15$ K (= 26 °C). Osmotic pressure corresponded to 0 (control), 2.5, 5.0, 7.5, and 10.0 MPa. Readings of conidial germination were assessed after 32 h of incubation at 26 °C with 12 h photophase. The 32 h interval was selected to facilitate spore germination in a sub-optimum environment.

2.6. Oxidative stress

Fungal tolerance to oxidative stress was assessed by adding hydrogen peroxide (30% v/v H_2O_2, Sigma Chemical Co., St Louis, MO, USA) to molten PDA medium (40–50 °C) at rates of 0 (control), 10, 20, 30, 40, 60, and 80 mM. Ten µL aliquots of suspensions containing 10^7 conidia/mL and controls in sterile 0.01% Tween 80 were placed on center of H_2O_2-amended PDA plates and incubated for 7 days at 26 °C with 12 h photophase. Since conidial germination did not occur within 48 h of exposure to H_2O_2-amended PDA at any tested concentration, we measured vegetative growth as the response variable. Colony growth radial diameter was determined on two perpendicular diameters of each colony with a digital caliper.

2.7. Statistical analysis for abiotic stress variables

Fungal growth parameters were assessed, based on reference to untreated controls. All experiments had three independent replicates per treatment and each experiment was repeated on three occasions to ensure reproducibility. Datasets for thermal and osmotic stress treatments were fitted to the non-linear 3-parameter logistic model, $y = d / [1 + \exp(b^*(\log(x) - \log(e))]$, whereas UV-B and oxidative stress data were fitted to the 3-parameter Gompertz model, $y = d^*\exp[-\exp(b^*(\log(x) - e))]$. For both models d is the upper limit, b the slope, x the concentration or exposure time, and e the median effective concentration (EC_{50}), median effective time (ET_{50}), median effective pressure (EP_{50}) or median effectivedose (ED_{50}). Model parameters were estimated by non-linear least squares using the R 'dcr' statistical package (Ritz and Streibig, 2005; R Core Team, 2014). Fittedfunctions were used to calculate the median effective concentration, dose, osmotic pressure or exposure time is the stress effect required to achieve 50% relative viability or vegetative growth in relation to unstressed controls. Relative viability was calculated as: Rv (%) = $(Vt / Vc)^*100$, where Vc is unstressed control viability and Vt is treatment viability.

2.8. Conidial production

Conidial production under solid-substrate fermentation was assessed for each fungal isolate. Two mL of conidial suspensions (5×10^6 conidia/mL) were inoculated on 20 g moistened, steamed parboiled rice (45% initial water content, w/w) in 125-mL conical Erlenmeyer flasks using methods outlined previously (Mascarin et al., 2013). This procedure delivered a final concentration of 10^6 conidia/g moistened rice. After 8 days incubation at 26 °C, 70–80% RH and 12 h photoperiod, sporulated rice was flooded with 100 mL sterile 0.1% v/v Tween 80 per flask for harvesting spores. The number of conidia/g rice was determined through serial dilutions followed by hemocytometer counts under 400X phase-contrast magnification. Each fungal isolate was replicated three times and the experiment conducted three times using new fungal inoculum. Conidial production data were $\log_{10}$-transformed prior to one-way analysis of variance (ANOVA), with fungal isolates separated by Tukey's HSD test using R (R Core Team, 2014).

2.9. Whitefly virulence bioassay

Each fungal isolate was bioassayed against *B. tabaci* using a ventilated leaf arena. The whitefly colony at Embrapa was identified as MEAM1 by molecular gene sequence markers from mtDNA cytochrome oxidase I (mtCOI) (Quintela et al., 2016). Bean plants were grown in 15-cm plastic pots containing 400 mL potting soil fertilized with 4-30-15 NPK (0.2 g/L) mixed with an organic substrate (Tropstrato®, Vida Verde Ltd., Mogi Mirim, SP, Brazil). Seedlings at the two-leaf stage were infested with 100 adults for a 24-h oviposition period. Plants were maintained in a screenhouse for 12 days. Leaves containing 2nd instars (F_1) were removed, transferred to agar-water ventilated plates (after Mascarin et al., 2013) and sprayed with 1 mL of conidial suspensions in 0.01% v/v Tween 80 using a Potter Spray Tower (Burkard Scientific Ltd., Uxbridge, UK). Four concentrations were tested, i.e., 10^5, 5×10^5, 2.5×10^6 and 1.25×10^7 conidia/mL providing deposition rates of 0.9×10^2, 4.5×10^2, 2.3×10^3 and 1.1×10^4 conidia/cm^2, respectively. Conidial viability assessed after 16 h on PDA at 26 °C was ≥ 98% germination for all isolates. Ventilated plates containing treated leaves were incubated at 26 °C, 70–80% RH, and 14 h photoperiod. Nymphal survivorship was assessed after five days. Dead nymphs were incubated inside Petri dishes lined with moist filter papers for four more days to inspect fungal growth and confirm mycosis. There were four replicate leaves per isolate/concentration, and the experiment was repeated three times with different fungal preparations and insects. Dose-response curves were fitted using the 3-parameter generalized log-logistic model

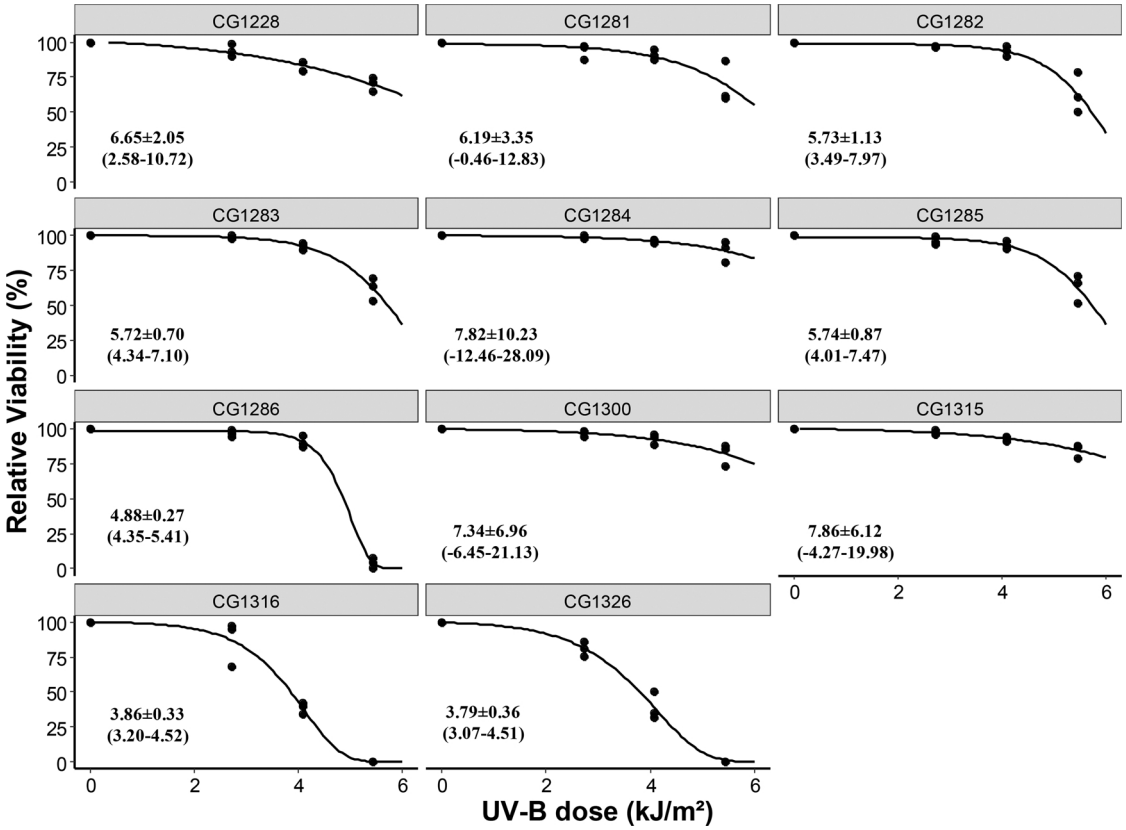

Fig. 3. Tolerance of *Cordyceps javanica* isolates to UV-B radiation. Data points show relative conidial viability on PDA after 48 h following variable exposures up to 5.44 kJ/m^2 and solid lines are fitted Gompertz (clog-log) models. Values inside plots correspond to predicted median effective doses (ED_{50} ± standard error) corresponding to 50% relative viability with 95% confidence intervals.

$$y = C + \frac{1-C}{(1 + exp(b(log(x)-log(e))))}$$

where y is proportional mortality, C is control mortality, b the slope of the dose-response curve, x the fungal concentration (conidia/ml), and e the LC_{50}. Dose-response curves were compared for parallelism among isolates using Wald χ^2 statistics at $P < 0.05$. LC_{50} values were compared with the reference isolate with the lowest value based on the relative potency (RP) test (Wheeler et al., 2007). Analysis was performed with R free statistical software (R Core Team, 2014) using the "drc" package (Ritz and Streibig, 2005).

2.10. Multivariate analysis

A multivariate principal component analysis (PCA) was performed to describe the correlation between response variables (i.e., phenotypic traits) and *C. javanica* isolates. Principal components (PCs) were loaded with response variables when correlation test yielded $r \geq 0.50$. Three first PCs accounting for > 70% of data variance were retained. Biplots (two-dimensional graph) using these three PCs correlating fungal isolates and response variables were built with the "FactoExtra" package (Kassambara, 2015) in the R platform. The analysis of hierarchical cluster (Sneath and Sokal, 1973) was estimated via the Euclidean distance between significant PCA variables with Ward's algorithm. The model was run with a bootstrap (n = 1000 replications) and significant clusters highlighted when attaining unbiased *P*-values (AUP ≥ 0.95). This analysis was run using "Pvclust" in R (Suzuki and Shimodaira, 2006) and results presented graphically (dendrogram), to identify groups of isolates with divergent characteristics.

3. Results

3.1. Molecular identification of Cordyceps isolates

All collected isolates were identified as *Cordyceps javanica* based on a consensus tree (Fig. 1). The partial β-tubulin genes from the 11 isolates were clonal and formed a single group clustering with the *C. javanica* type isolate. Morphology of conidial chains and absence of chlamydospores and synnemata were consistent with this finding (Duran et al., 2010). It is noteworthy that although CG1228 was originally isolated from the lepidopteran *R. albinella*, it formed a consistent phylogenetic group with the other ten CG isolates, originating from whitefly hosts (Fig. 1).

3.2. Heat shock stress

Conidial viability following heat shock varied among isolates ($\chi^2 = 389.4$, df = 30, $P < 0.0001$), with exposure time (treatment) described by a Gompertz (clog-log) model function (Fig. 2). Conidial survivorship was inversely proportional to heat exposure. Effective time to achieve 50% conidial viability (ET_{50}) among isolates varied from 0.48 (CG1286) to 1.25 h (CG1228 and CG1316) based on 48 h germination readings

3.3. UV-B stress

Susceptibility of *C. javanica* to UV-B radiation also varied among isolates and increased dosage ($\chi^2 = 366.1$, df = 30, $P < 0.0001$). CG1286, CG1316 and CG1326 failed to germinate after exposure to 5.44 kJ/m^2, while others retained ≥ 50% viability in the bioassay. CG1300, CG1284 and CG1283 were considered the most tolerant to

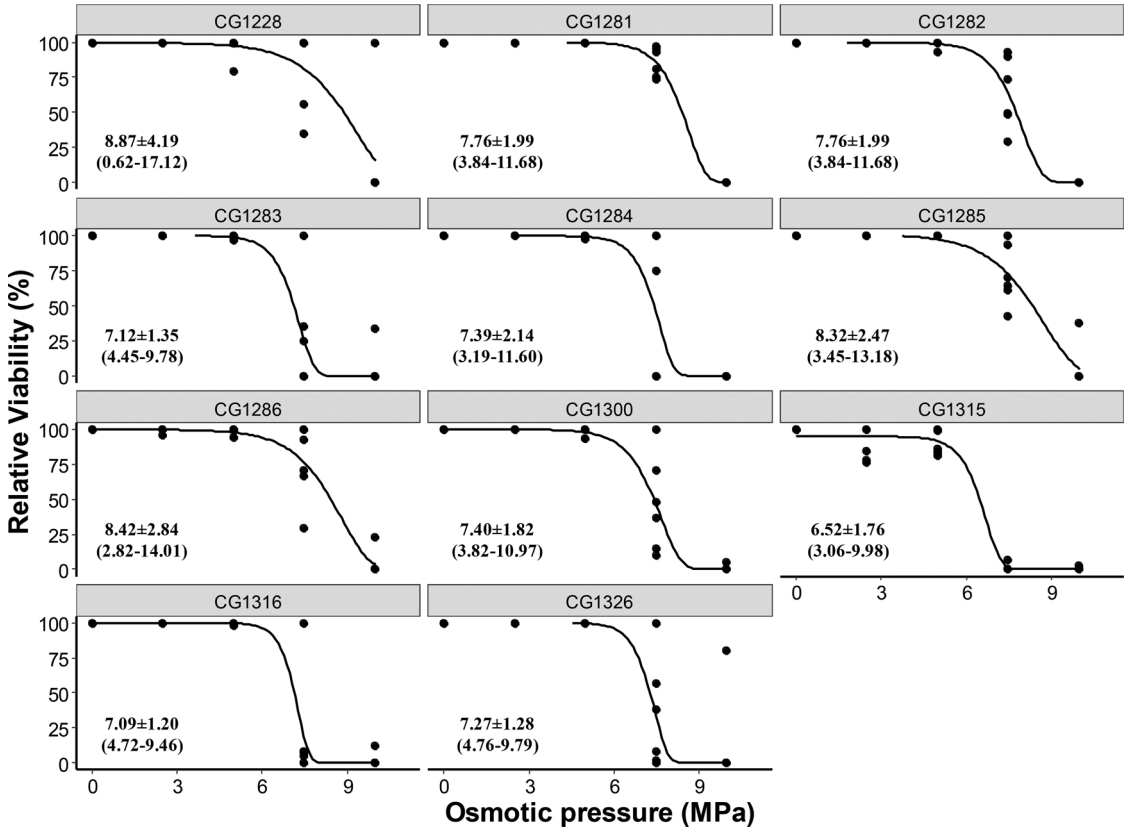

Fig. 4. Tolerance of *Cordyceps javanica* isolates to osmotic stress. Data points show relative conidial viability after 32 h incubation on PDA amended with NaCl to simulate increased osmotic pressures up to 9.95 MPa. Solid lines are fitted Gompertz (clog-log) models. Values inside plots correspond to predicted median effective pressures (EP_{50} ± standard error) corresponding to 50% relative viability with 95% confidence intervals.

UV-B radiation. Conidial viability following UV-B dosages and fitted with a Gompertz (clog-log) model revealed median effective doses (ED_{50}) values ranging from 3.79 (CG1326) to 7.86 (CG1315) kJ/m^2 after 48 h incubation (Fig. 3).

3.4. Osmotic stress

Susceptibility of *C. javanica* isolates to osmotic stress imposed by NaCl varied significantly ($\chi^2 = 158.5$, df = 30, $P < 0.0001$), and conidial viability dropped after 7.46 MPa in all cases (Fig. 4). A few isolates showed enhanced tolerance to osmotic stress treatments, although all failed to germinate effectively at the highest value (9.95 MPa). The response of conidial viability to increasing osmotic pressure fitted with a Gompertz (clog-log) model revealed ED_{50} values varying from 6.52 (CG1315) to 8.87 (CG1228) MPa at 32 h incubation.

3.5. Oxidative stress

Response of *C. javanica* to oxidative stress varied among isolates ($\chi^2 = 114.7$, df = 30, $P < 0.0001$), with viability rates decreasing with the increase in H_2O_2 concentration. Response to increasing oxidative stress over 7 days fitted with a Gompertz (clog-log) models showed ED_{50} values varying from 3.09 (CG1282) to 4.72 (CG1326) mM H_2O_2 (Fig. 5). At 8 mM H_2O_2, fungal growth was null in all cases.

3.6. Conidial production on solid substrate

Conidial yield after 8 days of solid-state fermentation (pre-cooked parboiled rice) varied among *C. javanica* isolates ($F = 16.86$, df = 11, 55, $P < 0.0001$). Mean separation tests revealed that two isolates in particular (CG1228 and CG1326) produced significantly more conidia (i.e., $> 7 \times 10^9$ conidia/g rice), when compared with the others (Fig. 6).

3.7. Virulence against whitefly

Mortality response of MEAM 1 nymphs varied among *C. javanica* isolates ($\chi^2 = 262.27$, df = 40, $P < 0.0001$) and followed a concentration-dependent relationship. with LC_{50} values between 1.10 and 3.03×10^6 conidia/mL. Isolate CG1316 was the most virulent, whereas CG1315 and CG1326 were significantly less virulent compared with the other isolates (Table 2). The negative slope values reflect relative virulence with the non-linear model, with greater negative values correlating with more virulent isolates.

3.8. Multivariate analysis

The variability in fungal isolate profiles to abiotic stress, virulence and spore production were best described by three principal components (PCs) which accounted for 74.5% of data variation, i.e., PC1 (30.8%), PC2 (24.0%) and PC3 (19.7%). The factor map (biplot) shows the groups of variables (arrows) denoting positive and negative correlations with each PC, with arrow length indicating the magnitude of each response for each PC (Fig. 7, Table S1). For example, PC1 was positively correlated with UV−B but negative with both oxidative and heat stress and conidial production (yield). By contrast, PC2 was positively correlated with osmotic stress but negatively correlated with heat stress and virulence, whereas PC3 was positively correlated with osmotic and UV−B stress and conidial yield but negatively correlated with oxidative stress. For example, osmotic stress variable showed the greatest contribution to PC2. Conversely, conidial yield had the greatest contribution to the variability of PC1 and PC3. Based on the quality of

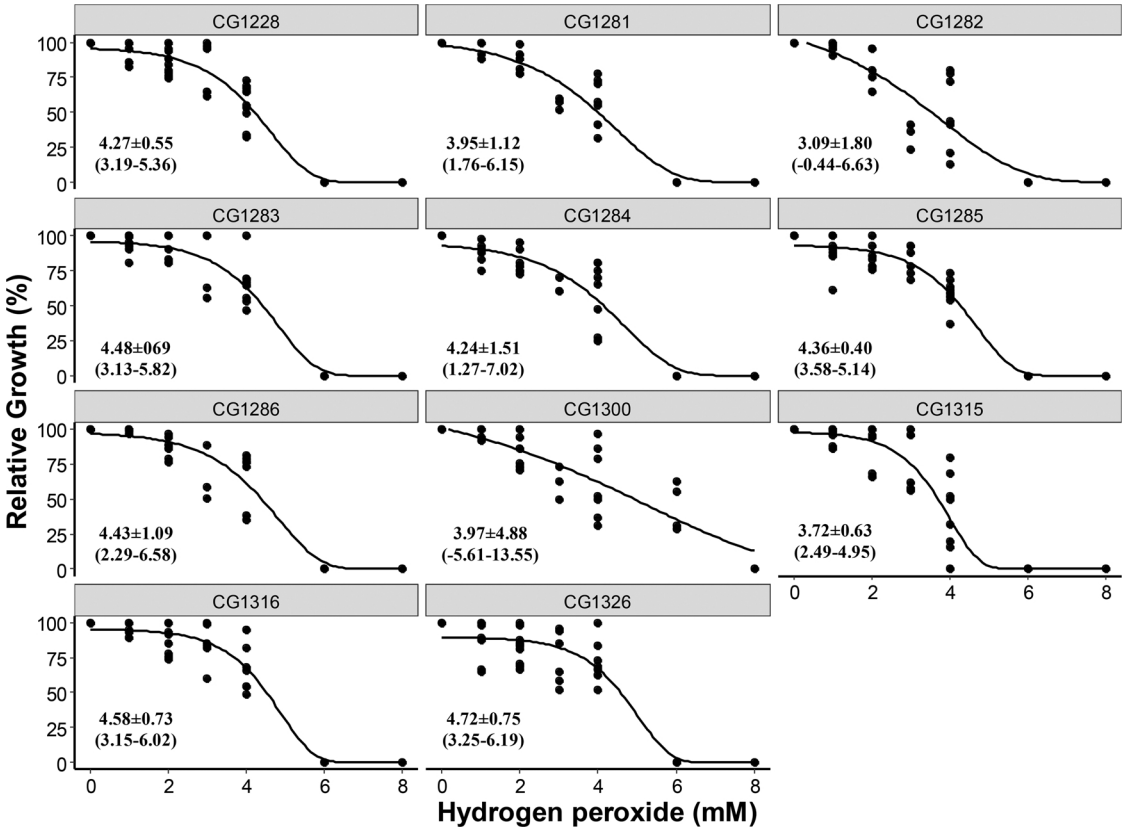

Fig. 5. Tolerance of *Cordyceps javanica* isolates to oxidative stress measured as relative vegetative growth (%) after seven days of incubation on PDA amended with H_2O_2 at concentrations from 0 (control) to 8 mM. At 8 mM H_2O_2, none of the fungi was able to grow. Values inside each plot correspond to predicted median effective concentrations (EC_{50} ± standard error) of which H_2O_2 reduced 50% of vegetative fungal growth followed by their 95% confidence intervals within brackets. Black dots represent experimental data and solid lines denote fitted Gompertz (clog-log) models.

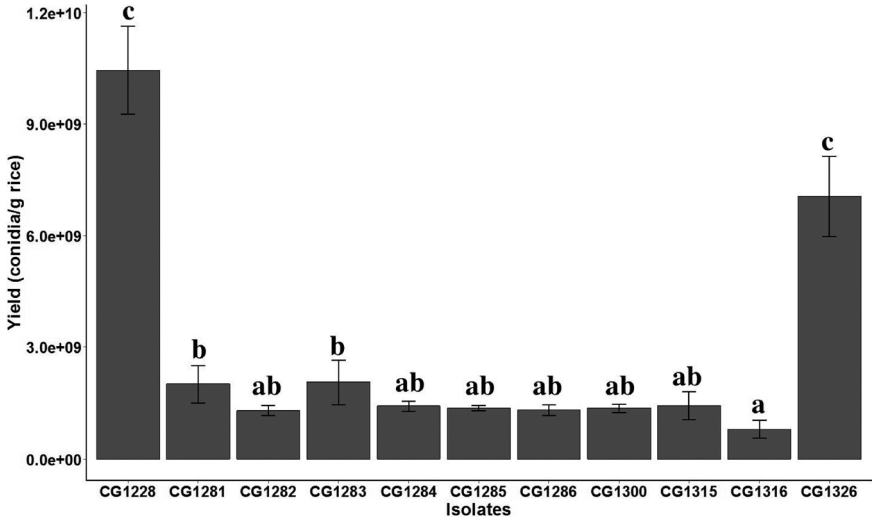

Fig. 6. Conidial production of *Cordyceps javanica* isolates per gram of rice (on dry basis) after 8 days of incubation at 26 °C with 12 h photoperiod. Bars are means (± standard error) from two independent experimental runs (n = 6). Different letters denote statistical differences (Tukey HSD, $P < 0.05$).

representation of fungal isolates measured by *cos*2 values, isolates CG1228, CG1286, CG1285, CG1315, CG1316 and CG1326 attained higher contribution (> 10%) to the PCs (Fig. 7, Table S1). Cluster analysis revealed three significant (AUP ≥ 0.95) groups formed by *C. javanica* isolates with shared phenotypes associated with (i) resistance to osmotic and oxidative stress, (ii) resilience to UV−B, and (iii) high virulence, conidial production and heat tolerance (Fig. 8). Isolate CG1228 performed well for oxidative tolerance, spore production and LC_{50}-based virulence. CG1283, CG1316, CG1228 and CG1326 were unrelated and did not group with the others.

4. Discussion

In nature, fungi are exposed to environmental stresses, with heat, UV radiation, nutritive, oxidiative and osmotic stresses among the most common (Rangel et al., 200, 2015; Duran et al., 2010). The ability of

Table 2
Estimates of 3-parameter log-logistic binomial model and median lethal concentrations (LC_{50}) and relative potency (RP) for concentration-mortality response of whitefly nymphs exposed to *Cordyceps javanica* isolates.

Fungal isolate	Model parameters			LC_{50} ($\times 10^6$ conidia/mL) [CI95%][b]	RP_{50} [*P*-value][c]
	Slope ± SE[a]	*c*	*e*		
CG1282	−0.86 ± 0.17	0.16	14.97	2.00 (1.24–3.24)	1.82 (0.07)
CG1281	−0.82 ± 0.09	0.15	14.74	1.60 (1.11–2.31)	1.45 (0.17)
CG1300	−0.66 ± 0.13	0.15	14.73	1.44 (0.81–2.54)	1.31 (0.45)
CG1284	−0.75 ± 0.12	0.16	14.87	1.70 (0.99–2.89)	1.55 (0.21)
CG1286	−0.99 ± 0.15	0.16	14.77	1.79 (1.28–2.50)	1.63 (0.06)
CG1285	−0.89 ± 0.11	0.08	14.63	1.80 (1.31–2.50)	1.64 (0.052)
CG1283	−0.89 ± 0.29	0.18	14.66	1.39 (0.47–4.15)	1.26 (0.77)
CG1315	−0.94 ± 0.17	0.09	14.92	2.46 (1.67–3.61)	*2.24 (0.006)
CG1316	−0.79 ± 0.09	0.04	14.02	1.10 (0.78–1.55)	1 (reference)
CG1326	−0.78 ± 0.12	0.10	15.19	3.03 (1.99–4.61)	*2.75 (0.0002)
CG1228	−0.83 ± 0.12	0.063	14.43	1.57 (0.99–2.47)	1.42 (0.23)

[a] All slopes were significantly different from 0 ($P < 0.01$) and the negative signal means that mortality curve increases with concentration, as the model is nonlinear.
[b] Median lethal concentration (LC_{50}) and confidence interval 95% (CI95%).
[c] Relative potency based on the lowest LC_{50} (CG1316) used as the reference for pairwise comparisons with other isolates (Wheeler et al., 2007). Asterisk (*) indicates significant *P*-value according to Student's *t*-test ($P < 0.05$).

entomopathogenic fungi to survive stress factors before infection, during cuticular penetration and *in vivo,* is a pre-requisite for successful biological control agents. Differences in environmental tolerances (UV-radiation and temperature) were reported among isolates of both *Beauveria* and *Metarhizium*, and linked with various geographic regions, arthropod hosts or rearing substrates (Braga et al., 2001; Fernandes et al., 2007, 2008, 2015; Lee et al., 2015). Phenotype differences among entomopathogenic fungi in response to oxidative stress during cuticular penetration, or when exposed to reactive oxygen species (ROS) induced by heat and UV radiation have been documented (Azevedo et al., 2014; Huarte-Bonnet et al., 2015). However, we are unaware of equivalent reports among *Cordyceps* (*Isaria*) spp.

We identified 11 isolates of *C. javanica* from two insect hosts in Brazil. Recent phylogenetic studies indicate that *C. javanica* is a generalist that infects many insects, but may have been formerly misidentified as *C.* (formerly *Isaria*) *fumosorosea* (Cabanillas et al., 2013; D'Alessandro et al., 2014; Gallou et al., 2016; Dunlap et al., 2017a, 2017b). For example, the isolate *C. fumosorosea* 'Apopka-97′, which is produced as a mycoinsecticide "PFR-97″ (Avery et al., 2013), is actually a *C. javanica* isolate. While all isolates were pathogenic to *B. tabaci* MEAM 1, we observed a 3-fold difference in LC_{50} values between isolates. Moreover, our results showed clear statistical differences based on tolerances to several environmental stresses, as well as conidial production under controlled laboratory conditions.

Stress responses and virulence of entomopathogenic fungi have a genetic basis, which is confirmed through gene deletion/knockout mutants and proteomics targeting enzymes or other proteins (Zhang et al., 2009; Wang et al., 2013; Ortiz-Urquiza and Keyhani, 2015). Moreover, the ability to genetically modify these fungi to enhance their virulence and tolerance to adverse conditions has been achieved (St. Leger and Wang, 2010; Zhao et al., 2016). However, to date most research selecting genes for improved stress tolerance and virulence concern just two species, i.e. *B. bassiana* and *M. anisopliae* sensu lato, with few reports for other hypocrealean species. Current regulatory policies pose a barrier to the commercial production or release of transgenic 'hypervirulent' isolates of entomopathogenic fungi for pest control (Gressel, 2001). Thus, selecting among endemic isolates for tolerances to specific environmental parameters such as UV-B and heat stress tolerances, as well as mass production characteristics, provides an approach to select candidate isolates to develop as mycoinsecticides. This approach can also serve as a basis for selection programs in which the goal is to generate phenotypes superior than their parental lineages (de Crecy et al., 2009).

Based on our principal component analysis, we hypothesize a trade-off among virulence, asexual (semelparous) reproduction and environmental stress tolerance for *C. javanica*, since no single isolate outperformed in all parameters evaluated in this study. Our results identified three clusters formed by isolates showing (i) resistance to osmotic and oxidative stress, (ii) resilience to UV-B, and (iii) high virulence, conidial-production and heat tolerance. Rangel et al. (2015) demonstrated osmotic and nutritive-stressed *Metarhizium robertsii* produced conidia with a robust stress phenotype and high virulence, but with low conidial yield in comparison to unstressed phenotype. It is likely that genetic traits involved in virulence share the same biochemical mechanisms to cope with stress tolerance or vice-versa, but there might be a cost that hinders semelparous reproduction in these fungi.

While a mycoinsecticide should be virulent to the target pest, the scalability of infective propagule production is important for selecting fungal isolates (Jaronski and Mascarin, 2016). Currently in the USA, two commercial products for *C. javanica* are based on blastospores produced from the isolate (Apopka-97) in liquid fermentation (Arthurs and Dara, 2018). Blastospores have benefits of low cost production and fast germination, but have more limited tolerances to environmental conditions when compared with conidial formulations (Jaronski and Mascarin, 2016). We did not assess the influence of nutritive media on growing blastospores of *C. javanica* in the present study; this needs to be documented in future research.

Based on our findings, isolate CG1228 might be a good candidate for a mycoinsecticide for whiteflies, while CG1315 might be a better candidate for use in a UV-intensive environment or region. The irradiation dosage in our tests (i.e., Quaite = 378 mW/m^2) was lower compared with the maximum recorded in São Paulo, Brazil, at solar noon in the summer (Quaite = 1300 mW/m^2) (Dias et al., 2018). These latter authors used a commercial solar chamber to compared UV-tolerances among a range of insect-pathogenic fungi under these maximum challenge conditions. In their test, an isolate of *C. javanica* originating from *B. tabaci* collected in Hawaii (ARSEF 3889) with an LT_{50} of 147 min at 1300 mW/m^2 was considered 'moderately UV-tolerant' when compared with other fungal species. Although differences in methodology makes direct comparisons difficult, the estimated LT_{50} dosage used by Dias et al. (2018) ($\approx$ 11.8 kJ/m^2 for ARSEF 3889) exceeded that of the most tolerant isolate in our tests (i.e., 7.9 kJ/m^2 for CG1315). However, we also note that germination may not be the best parameter to quantify UV-effects, since *in vitro* tests with conidia-inoculated agar medium exposed to UV-B radiation represent a 'worst-case scenario' for UV-inactivation (Fernández-Bravo et al., 2017). In tests from with *Metarhizium*-inoculated flies, these authors also demonstrated that UV-B radiation reduced fungal germination in vitro (i.e., in a Petri dish) far more readily when compared with corresponding reductions in virulence on the host cuticle.

We evaluated heat shock effects as a proxy for temperature tolerances. For instance, *in vitro* studies suggest that isolates producing more heat shock proteins might work better in hotter climates. Rangel and Roberts (2018) noted that an elevation of intracellular trehalose and mannitol content in *Metarhizium acridum* conidia confers higher intrinsic tolerance to UV-B radiation and heat. In studies with a Texas *Isaria* isolate, Cabanillas and Jones (2009) reported that while no growth occurred at 35 °C (upper limit), growth would resume when transferred to a permissive temperature (25 °C). However, no recovery or growth occurred after transfer from 40 °C to 25 °C. We suggest that our fungal isolates from the Cerrado (Brazilian's Savannah) should be optimized for the hot climate, and can potentially further be selected through directed evolution (de Crecy et al., 2009).

A limitation of our study is that isolates selected in laboratory bioassays (where barriers to infection are limited) may not necessarily be the best candidates under operational conditions; this would

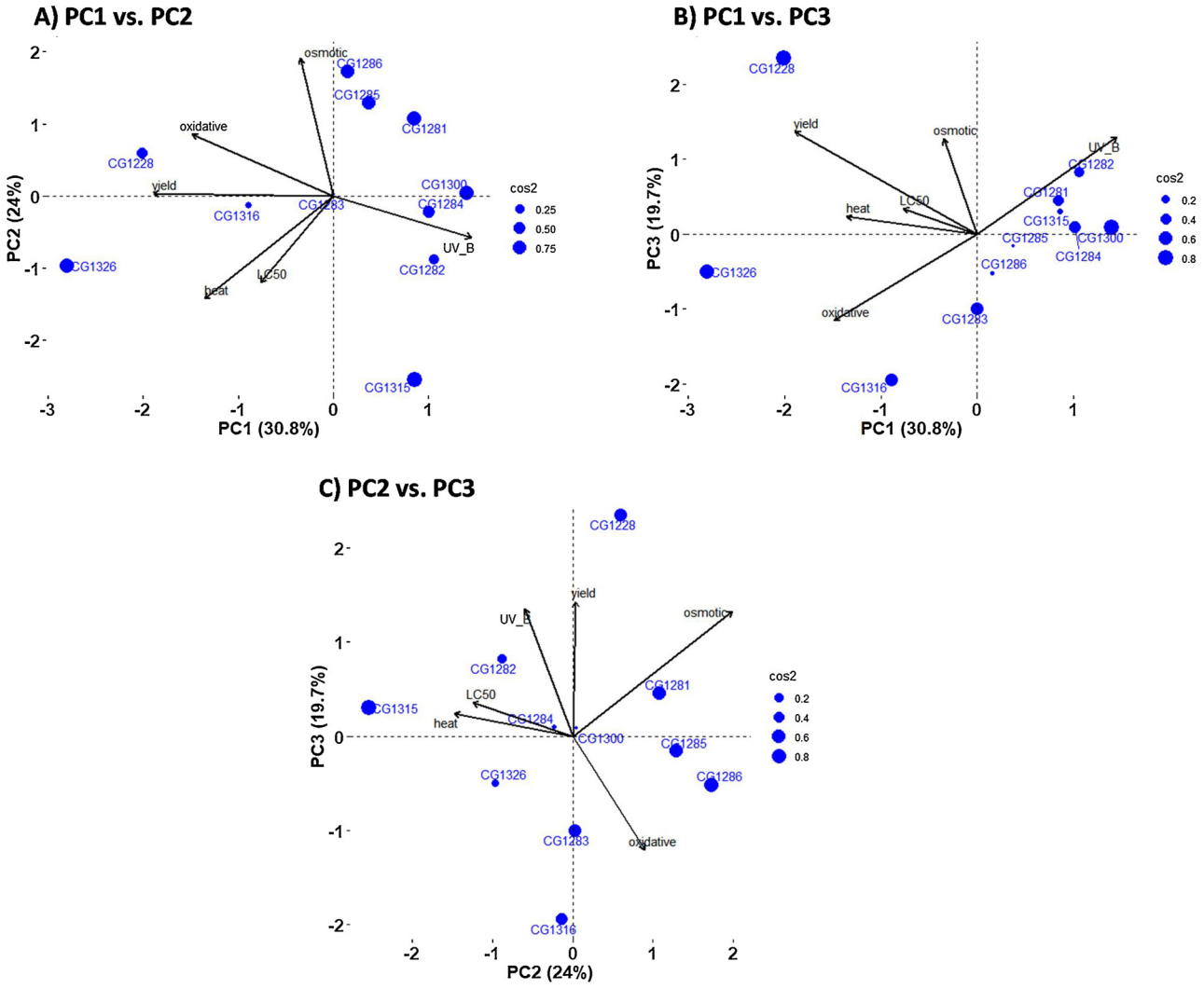

Fig. 7. Principal component analysis (PCA) explaining correlations between phenotypic traits (response variables) among 11 *Cordyceps javanica* isolates. Three principal components (PCs) accounted for 74.5% of data variance. Biplots (A–C) show correlations whereby (PC1) explained 30.8% of data variance and was positively loaded with UV-B stress, but negatively correlated with oxidative and heat stress and conidial yield. PC2 explained 24% of data variance and was positively loaded with osmotic stress, but negatively correlated with heat stress and virulence (LC_{50}). PC3 explained 19.7% of data variance and was positively loaded with osmotic and UV-B stress and conidial yield, but negatively loaded with oxidative stress. Arrows denote the direction of correlation, while arrow length represents the magnitude of the correlation squared (*cos*2). The size of circles (per isolate) represents their relative *cos*2 magnitude.

presumably vary and would need to be confirmed experimentally. Parameters known to influence the persistence and/or infectivity of entomopathogenic fungi under field conditions depend on microclimatic conditions (including temperature, humidity and radiation) in the specific insect habitat. For example, evaluations of *Lecanicillium muscarium* applied against the whitefly *Trialeurodes vaporariorum* in greenhouse tomato confirmed that the humid boundary layer underleaf surfaces supported high infection rates of nymphs, regardless of changes in ambient humidity inside the greenhouse (Fargues et al., 2005). Since field-screening trials of fungal isolates are costly and time-consuming, laboratory assays provide a useful starting point to identify promising isolates for field-testing.

In conclusion, selecting among isolates of entomopathogenic fungi will help support the development of new mycoinsecticides. In Brazil, whiteflies can build up large populations on soybean, bean, cotton and, more recently corn, grown under irrigation all year long (Quintela et al., 2016). Based on our data, several endemic isolates of *C. javanica* (especially isolate CG1228) are promising candidates for development as a whitefly biological control agent.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

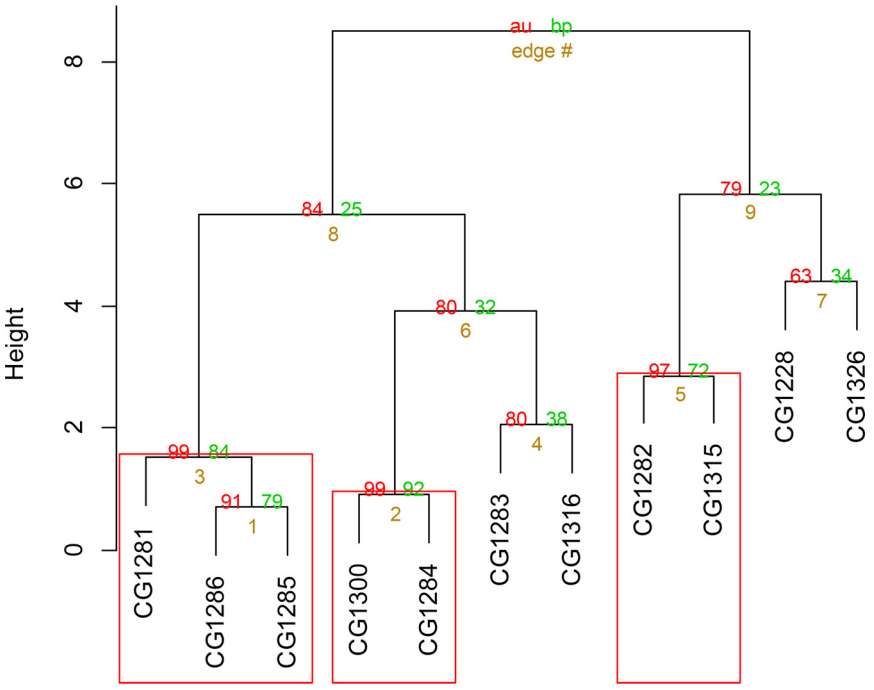

Fig. 8. Dendrogram showing hierarchical cluster of *Cordyceps javanica* isolates based on Euclidean distance between response variables (EC_{50}, ET_{50} and LC_{50} values and conidial production) and Ward's minimum variance algorithm. Values at branches are approximately unbiased *P*-values (upper left side), bootstrap probability *P*-values (upper right side), and cluster labels (bottom). Approximately unbiased *P*-values (AUP, %) ≥ 0.95 were computed by multiscale bootstrap resampling with n = 1000 replications. Significant clusters (AUP ≥ 0.95) highlighted by rectangles indicate high similarity across isolates.

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