Biological Control 66 (2013) 209–218

Contents lists available at SciVerse ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

The virulence of entomopathogenic fungi against *Bemisia tabaci* biotype B (Hemiptera: Aleyrodidae) and their conidial production using solid substrate fermentation

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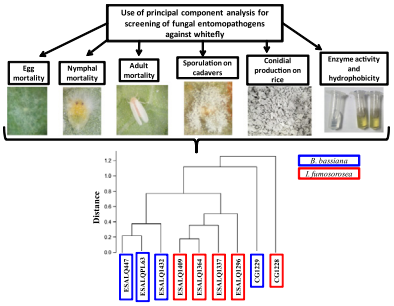
HIGHLIGHTS

- Higher virulence against nymphs was attained with *B. bassiana* and *I. fumosorosea*.
- None of the *B. bassiana* and *I. fumosorosea* isolates were effective against eggs.
- Whitefly adults are highly susceptible to *I. fumosorosea* isolates.
- Selection was based on virulence and sporulation on rice and nymphal cadavers.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 October 2012
Accepted 20 May 2013
Available online 25 May 2013

Keywords:
Beauveria bassiana
Isaria fumosorosea
Lecanicillium muscarium
Microbial control
Phaseolus vulgaris
Silverleaf whitefly

ABSTRACT

The virulence of five isolates of *Beauveria bassiana*, five of *Isaria fumosorosea* and four of *Lecanicillium muscarium* from Brazil was determined on whitefly *Bemisia tabaci* biotype B lifestages on bean leaves under laboratory conditions. The conidial yield (on cadavers or parboiled rice), surface hydrophobicity and enzyme activity were also determined. The isolates of *B. bassiana* and *I. fumosorosea* were the most virulent against nymphs (71–86% mortality within 8 d), with LT_{50} values ranging from 3 to 4 d after treatment with 10^7 conidia/mL (150 conidia/mm^2). Spore production on nymph cadavers reached 4–8 $\times$ 10^5 conidia/insect. The *L. muscarium* isolates demonstrated low virulence toward nymphs. After spraying eggs with 1 $\times$ 10^8 conidia/mL (1674 conidia/mm^2) of *B. bassiana* and *I. fumosorosea*, most nymphs hatched, but then 40–70% of these nymphs were infected by indirect exposure of conidia on the leaves. Adults exposed to treated leaf disks (150 conidia/mm^2) were more susceptible to *I. fumosorosea* than to *B. bassiana*. The enzyme activity (Pr1) and the relative conidial surface hydrophobicity were not correlated with any virulence parameter measured for either *B. bassiana* or *I. fumosorosea*. In addition, the highest conidial yields on parboiled rice using solid-state fermentation (4.9–11.4 $\times$ 10^9 conidia/g) were achieved by isolates of *I. fumosorosea* CG1228 and *B. bassiana* CG1229. *I. fumosorosea* CG1228 was highly virulent against whitefly nymphs and adults as well as attained high spore production on insect cadavers and parboiled rice. Our results indicate that *I. fumosorosea* CG1228 has desirable attributes for the development of a mycoinsecticide against *B. tabaci* biotype B.

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http://dx.doi.org/10.1016/j.biocontrol.2013.05.001

1. Introduction

Bemisia tabaci (Genn.) (Hemiptera: Aleyrodidae) is among the most devastating and widespread insect pest of a broad range of greenhouse and field crops worldwide. It is a serious threat to crop production due to direct damage and transmission of several plant viruses (Oliveira et al., 2001; Jones, 2003). *B. tabaci* is considered a cryptic species complex and was recently split into 11 groups encompassing 24 species (De Barro et al., 2011). The biotype B of *B. tabaci* (also known as Middle East-Asia Minor 1) is widely distributed throughout Brazil and it can cause economic losses estimated in 714 million US$/year in crops such as beans, soybeans, cotton, tomatoes, and leafy vegetables (Fontes et al., 2012; Oliveira et al., 2013). The control of this insect relies primarily on the use of chemical insecticides, but this stand-alone approach has encountered difficulties due to the selection of resistant individuals. In addition, the decreasing number of new registered insecticides, their harmful side effects to non-target organisms, the legal restrictions regarding their safe use and their environmental hazards have encouraged the adoption of additional control techniques, including biological control approaches.

Among several groups of biocontrol agents for whiteflies and other sap-sucking insects, entomopathogenic fungi possess the unique ability to infect their host directly through the integument. Moreover, they play a role in the natural mortality of whitefly populations (Lacey et al., 1996). The most promising mitosporic fungi include *Isaria fumosorosea*, *Lecanicillium* spp., *Beauveria bassiana* and *Aschersonia* spp. (Ascomycota: Hypocreales) (Faria and Wraight, 2001; Wraight et al., 2007; Lacey et al., 2008). *Lecanicillium* and *Aschersonia* species have been used to control whiteflies and related insects in greenhouses in Europe and Canada. These applications have been successful in cases where environmental conditions of high relative humidity and moderate temperatures are appropriate (Fransen et al., 1987; Chandler et al., 1994).

Several researchers evaluated fungal pathogens for the control of *B. tabaci* biotype B in the 1990's (Lacey et al., 2008). Despite considerable research in North America, Europe and Asia (Faria and Wraight, 2001; Lacey et al., 2008), little recent information exists on this topic in South America. Furthermore, little research on fungal entomopathogens of whiteflies has been conducted in Brazil (Sosa-Gómez et al., 1997; Lourenção et al., 1999). Currently, the authors are unaware of any commercial and registered mycoinsecticides available against whiteflies in Brazil.

The screening of fungal isolates for virulence characteristics is of paramount importance for the success of biocontrol strategies toward whiteflies and other insects (Faria and Wraight, 2001; Lacey et al., 2008). Virulence can be indirectly measured with the activities of enzymes related to infection pathways, such as subtilisin-like (Pr1) and trypsin-like (Pr2) enzymes and chitinases (Shah et al., 2005, 2007; Jackson et al., 2010). In addition to virulence, other important parameters should be considered for commercial development of mycoinsecticides. For example, the production of infective and stable propagules on inexpensive artificial substrates is a requirement for industrial-scale production with either solid-state or submerged liquid fermentation methods (Jackson et al., 2010). In some cases the ability of the fungus to sporulate on host cadavers may also be beneficial for secondary infections and extended control.

In this study, the virulence of isolates of three genera of hypocrealean anamorphic fungi (*Beauveria, Isaria,* and *Lecanicillium*) was determined on eggs, nymphs and adults of *B. tabaci* biotype B. The relationship between surface hydrophobicity, spore-bound Pr1 enzyme activity and the virulence factors of selected isolates was investigated. We also determined the yield of conidia obtained from whitefly cadavers and on parboiled rice using solid-state fermentation.

2. Materials and methods

2.1. Insect colony

The *B. tabaci* biotype B colony originated from bean, soybean and tomato crops at the EMBRAPA Rice and Beans Research Station in Santo Antônio de Goiás, GO, and Campinas, SP, Brazil. Whiteflies were mass-reared in a screenhouse (9 × 8 m) on butter bean (*Phaseolus lunatus* L.), common bean (*P. vulgaris* L., cv. Pérola), and soybean (*Glycine max* L., cv. Favorita). Biotype B was identified using a specific molecular marker (SCAR, Sequence Characterized Amplified Regions) (Queiroz da Silva, 2006).

2.2. Fungal preparations

Five isolates of *B. bassiana*, five of *I. fumosorosea* and four of *L. muscarium* originating from infected *B. tabaci* and other hosts from diverse sites of Brazil were obtained from the Invertebrate Fungal Collection at EMBRAPA Genetic Resources and Biotechnology (Brasília, Brazil) and the Entomopathogenic Fungal Collection at ESALQ-University of São Paulo (Piracicaba, Brazil) (Table 1). The identity of Brazilian isolates of *B. bassiana* and *I. fumosorosea* was confirmed by molecular analysis using the nucleotide sequence of the divergent domain (d1/d2) at the distal end of the 26S rRNA gene. Prior to use in studies, all fungal isolates were passaged through whitefly nymphs and subcultured on SDA + yeast extract (SDAY) no more than four times to avoid attenuation of virulence (Butt and Goettel, 2000; Shah et al., 2007). All assays utilized conidia inoculum harvested from 14-d-old cultures and suspended in 0.01% (v/v) surfactant (Tween 80®, Vetec Química Fina Ltda., Rio de Janeiro, RJ, Brazil). Conidial viability was determined after 16 h at 26 °C on 1% (w/v) water agar at 400× magnification. The conidial viability of all isolates exceeded 85% germination.

2.3. Bioassays against whitefly nymphs

Whiteflies were obtained from bean plants (*P. vulgaris*, cv. Pérola) grown in plastic pots (450 mL) filled with organic substrate amended with mineral nutrients (Tecnutri®, Itatiba, SP, Brazil) and maintained in a screenhouse (9 × 8 m). To generate infested leaves for bioassay, pest free plants (15–20 d old with two primary leaves) were placed near the adult-infested plants for 24 h. This procedure provided more than 40 eggs per leaf. The adult whiteflies were removed and newly infested plants moved to another screenhouse for 11–13 d, until nymphs reached the 2nd instar (0.30–0.44 mm in length and 0.18–0.36 mm in width) (Quintela, 2004). The nymph-infested leaves were excised and placed with ventral (abaxial) surface up on 14 mL of molten water agar (1% w/v) (Merck®) in polystyrene Petri plates (100 × 20 mm) with lids containing a 4-cm diameter central holes covered with cheesecloth (30-μm pore mesh) to allow ventilation.

For bioassays, 40–60 nymphs on ventral surface of excised bean leaves were previously marked with a small black dot nearby their position and then sprayed with conidia from each of the 14 isolates using a micro-spray tower (Mascarin et al., 2013). Conidia were harvest from SDAY plates with a microbiological loop and immediately suspended in 10 mL of sterile aqueous solution of 0.01% (v/v) Tween 80 into 50-mL plastic centrifuge tubes. The suspension was vigorously agitated on vortex for 2 min and filtered through two layers of 30-μm pore-sized nylon cheesecloth. The filtered suspension (10 mL) was agitated again for 1 min before application and conidial concentrations were enumerated by hemocytometer (Brightline Improved Neubauer, New Optik®, Brazil) at 400× magnification. In each case a 3 mL aliquot of each suspension was applied at 1×10^7 conidia/mL resulting in a volume application rate

Table 1
Brazilian entomopathogenic fungal species, strain codes, hosts/substrates and places of origin.

Fungus	Strain code[a]	Host/source	Collection site
Beauveria bassiana	ESALQ-PL63	*Atta* spp. (soil)	Piracicaba-SP
	ESALQ447	*Solenopsis invicta* (soil)	Cuiabá-MT
	ESALQ1432	*Diaphorina citri* (jasmine plant)	Piracicaba-SP
	ESALQ-CB66	*Hypothenemus hampei* (coffee)	São José do Rio Pardo-SP
	CG1229	*Rupela albinella* (rice)	Arari-MA
Lecanicillium muscarium	ESALQ 972	*Coccus viridis* (coffee)	Piracicaba-SP
	ESALQ 1351	*Praelongorthezia praelonga* (citrus)	São Paulo state
	ESALQ 1410	*B. tabaci* (cucumber)	Piracicaba-SP
	ESALQ 1408	*B. tabaci* (cucumber)	Itapetininga-SP
Isaria fumosorosea	ESALQ 1296	*B. tabaci* (unknown)	Jaboticabal-SP
	ESALQ 1337	*B. tabaci* (greenhouse colony)	Piracicaba-SP
	ESALQ 1364	*Myzus persicae* (collard)	Piracicaba-SP
	ESALQ 1409	*B. tabaci* (cucumber)	Itapetininga-SP
	CG1228	*R. albinella* (rice)	Arari-MA

[a] ESALQ means that fungi are maintained in ESALQ-University of São Paulo (Piracicaba, SP, Brazil), whereas CG is the code for fungi preserved in EMBRAPA Genetic Resources and Biotechnology (Brasília, DF, Brazil).

of approximately 2.15 μL/cm^2 (≈215 L/ha) and deposition rate of 150 ± 34 conidia/mm^2 (95% confidence limits: 34–658 conidia/mm^2, n = 21) on the leaf surface. Controls consisted of nymphs sprayed with the carrier surfactant solution (0.01% Tween 80) and unsprayed nymphs (e.g., untreated control), where the latter accounted for natural mortality. There were 5 replicates (plates) per fungal isolate (150–300 nymphs per treatment). After spraying, the leaves were allowed to air dry prior to incubation. The treated plates were placed upside down (inverted) on wire racks, so that the ventilation holes were not blocked, and held in a growth chamber at 26 ± 0.5 °C, 80 ± 11% relative humidity (RH) with a 14:10 (L:D) h photoperiod. Nymphal mortality was monitored for 8 d under a dissecting stereoscope (40×). Dead nymphs became desiccated or developed yellowish (*L. muscarium* and *I. fumosorosea*) or reddish (*B. bassiana*) symptoms with mycelial or conidial growth. All non-sporulated nymphs were incubated on 1% water agar for 2–3 d to confirm infection status at 26 ± 0.5 °C with a 12:12 (L:D) h photoperiod. All 14 fungal isolates were assayed together per experiment. The entire experiment was carried out three times (n = 3 true replicates) on different days (randomized complete block design with days as block) using different batches of conidial suspensions and different generations of insects per experiment.

2.4. In vivo sporulation

Comparisons of conidial yield was assessed from 4th instar nymphs. In each case, 60 freshly mycosed (non-sporulating) cadavers were randomly removed from each fungal treatment, surface sterilized with 70% ethanol for 1 min, rinsed twice in sterile deionized water for 30 s. Then cadavers were placed on wet filter paper and incubated inside a sealed Petri dish (90 × 15 cm) maintained at 26 ± 0.5 °C, 14:10 (L:D) h photoperiod and 100% RH. To quantify yield, after 6 d four randomly selected cadavers were placed in 1.8-mL microcentrifuge tubes (Eppendorf®) containing 0.5 mL of 0.1% Tween 80 and vigorously vortexed for 1 min. The cadavers were macerated with a glass pestle for 30 s to enhance dislodgement of conidia. Conidial yields were calculated based on hemocytometer counts. Four replicate assays were used for each fungal isolate (minimum of 16 cadavers per treatment), and the experiment was repeated twice on different days (randomized complete block design with days as block). In all, sporulation was measured on 448 cadavers (14 isolates × 8 replicates × 4 cadavers).

2.5. Bioassays with eggs and residual activity against hatched nymphs

Bean leaves (cv. Pérola) containing less than 48-h-old eggs were excised from plants and placed on 1% water agar plates. The surface of the leaf was marked with a permanent ink pen near to 40–60 eggs per leaf. The infested leaves were placed in ventilated incubation plates, as described above, and sprayed with suspensions containing 1 × 10^8 conidia/mL of five *I. fumosorosea* and four *B. bassiana* isolates. The spray deposition on leaf surface delivered a final rate of 1674 ± 261 conidia/mm^2 (95% CL: 397–7060 conidia/mm^2, n = 21). The control eggs on leaves were sprayed with 0.05% solution of Tween 80. The excised leaves were incubated ventral side down on wire racks in a growth chamber at 26 ± 0.5 °C and 87 ± 11% RH with a 14:10 (L:D) h photoperiod. Each treatment comprised four to five replicate plates (minimum of 160 eggs per treatment). All treatments were tested in the same assay. The proportion of egg mortality was measured by comparing the number of unhatched eggs to the total number of initially marked eggs. Furthermore, the eggs with signs of mycosis were scored as sporulated eggs. At the same experiment, after 8 d incubation period, 40–50 late 1st and early 2nd instar nymphs were selected and marked on the same treated leaves for all treatments. To determine the residual activity of the fungi against the hatched nymphs, three days later the number of dead nymphs was recorded. The experiment was repeated three times on different days (randomized complete block design with days as block).

2.6. Virulence of best fungal isolates against adults

Whitefly adults (⩽5 days post-emergence) were exposed to leaf disks containing spray residues of leading fungal candidates. Leaf disks (3.8 cm, in diameter) were treated with 1 × 10^7 conidia/mL (=150 conidia/mm^2) prepared in a sterile 0.05% Tween 80 solution for each fungal isolate using the micro-sprayer as explained above. Leaf arenas from the control groups were treated with a sterile solution of Tween 80 (0.05%). After spraying, leaf disks were allowed to air dry and then placed on 8 mL of 1% molten water agar in acrylic vials (77 × 38 mm). The adults were captured in glass vials, transferred to the treated leaf arenas and confined using cheesecloth tissue (30-μm pore mesh) affixed with a rubber band. Each vial contained approximately 25–40 individuals. The vials were placed top side down on wire racks and held in a growth chamber at 26 ± 0.5 °C and 80 ± 11% RH with a 14:10 (L:D) h photoperiod. Each fungal isolate had six leaf disks (replicates) and all isolates were tested at the same time per assay. The bioassay was conducted twice using a randomized complete block design with test date serving as block. The proportion of fungus-killed adults and sporulated cadavers were recorded 7 d post-inoculation. The remaining non-sporulated cadavers were also collected and transferred to humid chambers to confirm mycosis. The sporulated cadavers were recorded on the leaf arenas, on the vial walls,

and in the humid chambers. All these data were pooled together to calculate the total percentage of sporulation.

2.7. Surface hydrophobicity measurements

The conidial surface hydrophobicity was examined using an aqueous-solvent partitioning assay that determines the ratio of conidia distributed in the aqueous or organic phases. The organic phase used was hexane (Sigma–Aldrich®). The conidia from nine sporulated fungal isolates grown for two weeks on SDAY plates were harvested and suspended in 0.1 M KNO_3 solution and adjusted to a desired concentration of 1×10^7 conidia/mL, using a modification of Shah et al. (2007). Three milliliter of spore suspension was transferred to a glass vial, and 300 μL of hexane was added. Each isolate had three vials (replicates) prepared with different fungal stock cultures. The vials were agitated for 1 min. The suspension was left for 15 min to allow phase separation, after that the solvent phase was removed. The final conidial concentration in the aqueous phase (D_{aq}) was recorded. Percent relative hydrophobicity was determined as $100\times$ [1 – (D_{aq}/D_{total})], where $D_{(total)}$ and $D_{(aq)}$ represent the values of the starting sample and resulting aqueous phase, respectively.

2.8. Enzyme assay for spore-bound Pr1

The activity of spore-bound Pr1 was determined for preselected isolates of *I. fumosorosea* and *B. bassiana*. The procedure followed Shah et al. (2007) with slight modifications. Ten milligram of freshly harvested conidia grown on SDAY plates were incubated in 1 mL 0.1 M Tris–Cl (pH 7.95) amended with 1 mM succinyl-ala-ala-pro-phe-p-nitroanilide ($C_{30}H_{36}N_6O_9$) (Sigma-Aldrich®) for 5 min at room temperature (26 ± 1 °C). After incubation, conidia were separated from the aqueous phase by centrifugation at 12,000*g* (Z324 K, Hermle® centrifuge) for 10 min at 4 °C. The yellowish aqueous phase (100 μL) located under the conidial mass was transferred to wells in a flat-bottom microtiter plate, and the absorbance at 405 nm was measured using a spectrophotometer (model Epocha, Biotek®). There were four replicates for each isolate. Buffered substrates were used as controls. The enzymatic cleavage of the 4-nitroanilide substrate yields 4-nitroaniline (yellow color under alkaline conditions). Therefore, to convert an absorbance value into Pr1 activity expressed as μmol/mL/min, we used the molar extinction coefficient of 8800 $M^{-1}cm^{-1}$ for 4-nitroanilide, given by the manufacturer.

2.9. Conidial production using solid-state fermentation

Conidial yields of *B. bassiana*. and *I. fumosorosea* isolates grown on autoclaved parboiled rice were compared. Twenty grams of moistened and autoclaved rice were placed in a 125-mL conical Erlenmeyer flask, capped with a cotton ball and aluminum foil, and inoculated with 2 mL of conidial suspension, which delivered 10^7 conidia/g of wet rice. The water content was 44.0 ± 0.3% (e.g., ~11.2 g of dried rice). Flasks were incubated for 11 d at 26 °C with a 14:10 (L:D) h photoperiod. There were three replicates per fungal isolate. Conidial yields were assessed through agitation of cultures with sterile 0.1% Tween 80 solutions at 200 rpm for 1 h in an incubator shaker (TE-420, Tecnal®, Brazil) at 27 ± 1 °C. A 0.1-mL aliquot taken from the center of each conidial suspension was diluted (1000×) in a 0.1% Tween 80 solution. The amount of conidia produced per gram of rice on wet weight basis (20 g/flask) was determined through hemocytometer counts. Conidial viability was assessed from 200 conidia 16 h after inoculating 200 μL of 1×10^6 conidia/mL on 1% water agar plates (Rodak®, 60 × 15 mm) at 26 °C. All fungal isolates were assayed together and the experiment was repeated twice on different times using different batches of fungal inoculums.

2.10. Statistical analyses

All the data sets were previously checked for normality and homoscedasticity with Shapiro–Wilk and Brown–Forsythe tests and with diagnostic residual plots. Proportional (categorical) data were analyzed using logistic regression in the generalized linear mixed model (GLMM) with binomial distribution and logit link function (Jaeger, 2008). On the other hand, the continuous or non-binomial data that did not match linear modeling assumptions were transformed to normalize variances prior to analysis of variance (ANOVA). All analyses were conducted in SAS software, version 9.2 (SAS, 2008).

In all tests, fungal virulence was expressed and compared in terms of percent mortality at different lifestages and survivorship. For experiments conducted over time, the test dates were considered as experimental blocks, but block interactions were not tested due to potential restriction error (Sokal and Rohlf, 1995). The proportional (binomial) data of total nymph mortality, hatched nymph mortality, egg mortality, sporulated eggs, adult mortality and sporulated adults were fitted using logistic regression in GLMM with experimental blocks as a random effect in the mixed logit model (PROC GLIMMIX). If overdispersion was found in the data (e.g., residual deviance greater than 1), it was included in model as another random effect (Jaeger, 2008). *F*-statistics were derived from Wald-type III test for fixed effect in the mixed logit model (e.g., fungal isolates) and significantly different means were identified by Tukey's honestly significant difference (Tukey's HSD) test at $P \leqslant 0.05$. For non-binomial data, such as the number of conidia produced per cadaver and conidial production on parboiled rice, we used the square-root transformation prior to ANOVA. Then, treatment (e.g., fungal isolates) means were separated by Tukey's HSD test at $P \leqslant 0.05$. The survival analysis based on the Kaplan–Meier product-limit method calculated in the LIFETEST procedure in SAS was used to determine survival probability functions as well as to estimate the median time to death (LT_{50}, the time required to kill 50% of the treated insects) for whitefly nymphs exposed to different fungi with censored data for insects surviving beyond 8 d incubation period. The survival (function) curves for different fungal treatments were compared by the log-rank chi-squared test with *P*-values adjusted by Tukey's HSD and considering a significance level of 5% (Allison, 1995). Furthermore, estimated LT_{50} values were compared by the overlap of their 95% confidence intervals (95% CI). All data averages in the figures and tables are shown as untransformed values.

A principal component analysis (PCA) was used to identify the best isolates of *B. bassiana* and *I. fumosorosea* using seven out of the 11 measured response variables (categories) related to virulence factors, conidial production on cadavers and conidial production on rice. The seven preselected variables showed significant differences between the fungal isolates, whereas the others were not included in PCA (relative hydrophobicity, egg mortality, sporulated eggs, and Pr1 activity). The PCA was performed with the package FactoMineR in R 2.15.2 (R Development Core Team, 2012) software. Since the variables were measured in different scales, the PCA was performed with standardized data (mean = 0, variance = 1). The principal axis method was used to extract the components, and this was followed by varimax (orthogonal) rotation to identify the observed variables that demonstrated high loading for each component retained in the analysis. The eigenvalues of the principal components represented a measure of their associated variance, and the contribution of each observable variable in these components was given by the loadings (Lê et al., 2008). A Pearson's correlation coefficient was estimated to test

for correlations between individual components and variables; hence, each component was described only by the significant variables at a significance threshold of $P \leqslant 0.15$. Biplot graphs were drawn based on the principal components that explained at least 70% of the total variance to aid in the interpretation of results. In addition, a cluster analysis was performed with the unweighted pair-group with arithmetic mean (e.g., average UPGMA) method to calculate the Euclidean distances of dissimilarity (Everitt and Hothorn, 2010). This method provided the number of clusters formed among the *B. bassiana* and *I. fumosorosea* isolates on the basis of the meaningful observed variables derived from PCA. Highest similarities between isolates were scored as having the shortest distances.

3. Results

3.1. Virulence of fungal entomopathogens to whitefly nymphs

All fungi were pathogenic to whitefly nymphs at 1×10^7 conidia/mL (150 conidia/mm^2) ($F_{15,209} = 26.67$, $P < 0.0001$) (Fig. 1). Infected nymphs began to die 2 d after exposure to conidia. The average mortalities of the unsprayed insects and insects sprayed with Tween 80 (0.01%) were 2.0 and 6.2% after 8 d, respectively, and 88.4–90.7% of these nymphs turned into adults. Isolates ESALQ1409 and ESALQ1337 of *I. fumosorosea* and only CG1229 of *B. bassiana* were significantly more virulent to nymphs than all the *L. muscarium* isolates, causing nymphal mortalities >77%; but these isolates were as virulent as the isolates ESALQ-PL63, ESALQ1432 and ESALQ447 of *B. bassiana* and ESALQ1296, ESALQ1364 and CG1228 of *I. fumosorosea* (Fig. 1). The percentage of sporulation on nymphal cadavers was >90% for all fungal isolates (data not shown). Estimated LT_{50} values showed that most *B. bassiana* and *I. fumosorosea* isolates killed whiteflies faster (3–5 d) compared with *L. muscarium* isolates ($\geqslant$6 d) (Table 2). *I. fumosorosea* ESALQ1364 attained the lowest LT_{50} value (3 d). All fungal treatments decreased survival rates of whitefly nymphs (Log-rank: $\chi^2_{15} = 2625.3$, $P < 0.0001$). *I. fumosorosea* and *B. bassiana* isolates also killed more quickly the nymphs than the *L. muscarium* isolates ($P < 0.05$), with the exception of *B. bassiana* ESALQ-CB66 (Fig. 2).

Table 2
Virulence (LT_{50}) of different fungal isolates to 2nd instar nymphs of *B. tabaci* biotype B.

Fungus	Isolate	LT_{50}[a] (± SE)	95% CI[b]	
			Lower	Upper
L. muscarium	ESALQ1408	6 ± 0.1	5.8	6.2
	ESALQ1410	8 ± 0.2	7.7	8.3
	ESALQ972	8 ± 0.1	7.7	8.3
	ESALQ1351	NE[c]	–	–
I. fumosorosea	CG1228	4 ± 0.1	3.9	4.1
	ESALQ1296	4 ± 0.1	3.9	4.1
	ESALQ1337	4 ± 0.1	3.9	4.1
	ESALQ1364	3 ± 0.1	2.9	3.1
	ESALQ1409	4 ± 0.1	3.9	4.2
B. bassiana	CG1229	4 ± 0.1	3.9	4.1
	ESALQ1432	4 ± 0.2	3.7	4.3
	ESALQ447	6 ± 0.1	5.8	6.2
	ESALQ-PL63	5 ± 0.1	4.7	5.3
	ESALQ-CB66	6 ± 0.1	5.7	6.3

[a] LT_{50} values for mortality were estimated by survivorship analysis (Kaplan–Meier product-limit method) with censored data for insects surviving >8 d incubation period.
[b] 95% Confidence intervals that did not overlap indicate differences between LT_{50} values.
[c] Nonestimable: nymphal survival exceeded 50% and, therefore, precluded estimation of LT_{50} value.

3.2. In vivo sporulation

Quantitative estimates of in vivo sporulation varied among isolates ($F_{13,97} = 7.44$; $P < 0.0001$), ranging from 0.9 to 7.9×10^5 conidia/cadaver (Fig. 3). Highest inoculum productions were achieved by *B. bassiana* ESALQ-CB66 and CG1229 although these isolates were not statistically different from those that produced $\geqslant 3.3 \times 10^5$ conidia/cadaver. From a practical viewpoint, it would be necessary from nine to 14 sporulated cadavers to provide inoculation similarly to the rates applied to the excised leaves (i.e. 150 conidia/mm^2). Three out of four *L. muscarium* isolates and *I. fumosorosea* (ESALQ1296) resulted in $\leqslant 1.8 \times 10^5$ conidia/cadaver.

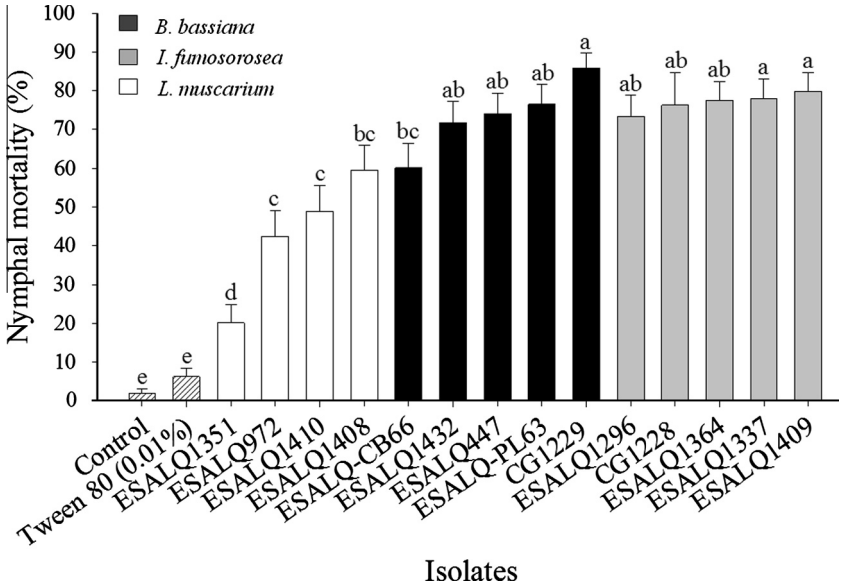

Fig. 1. Mortality of *B. tabaci* biotype B nymphs treated with 150 conidia/mm^2 (=1×10^7 conidia/mL) of *B. bassiana*, *I. fumosorosea* and *L. muscarium* isolates at 8 d post-inoculation. Error bars represent the standard error of the mean total mortality. Bars indicated by the same letters are not significantly different (Tukey's HSD, $P \leqslant 0.05$).

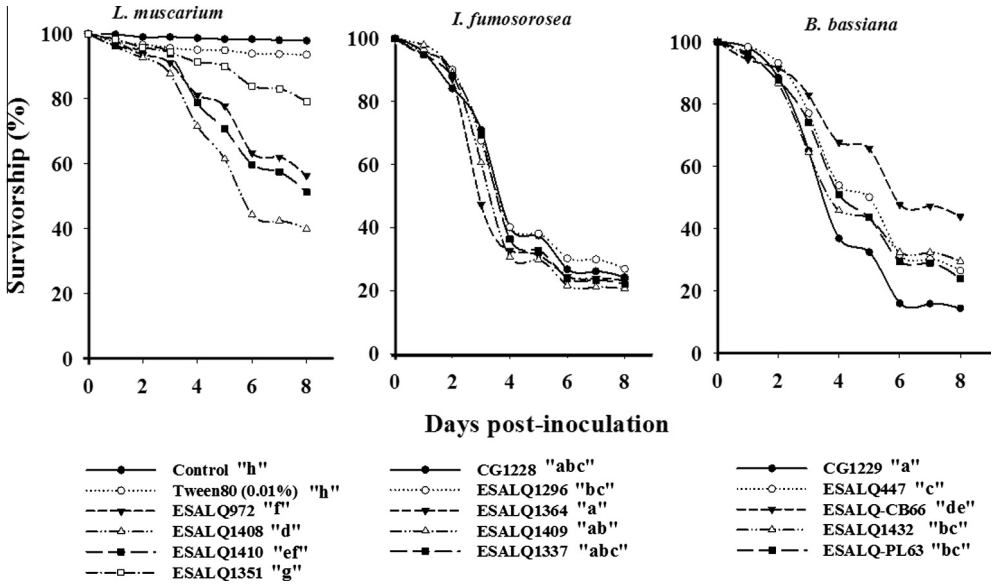

Fig. 2. Survival probability curves of 2nd instar nymphs of *B. tabaci* biotype B over time after exposure to *B. bassiana*, *I. fumosorosea* and *L. muscarium* isolates applied at 150 conidia/mm^2 (=1 × 10^7 conidia/mL). Survival curves followed by the same letters, in quotes, were not significantly different by log-rank test with *P*-values adjusted by Tukey's HSD ($P \leqslant 0.05$).

Interestingly, there was a positive relationship between the overall nymph mortality and the conidial production from cadavers (Spearman's correlation: $r = 0.56$, $P = 0.039$).

3.3. Bioassays with eggs and residual activity against hatched nymphs

Whitefly eggs had low susceptibility to all fungal isolates, with >91% nymphs successfully emerging from eggs treated with the highest fungal application rate (1674 conidia/mm^2 = 1 × 10^8 conidia/mL), and no statistical treatment effect observed ($F_{10,194} = 0.61$, $P = 0.78$). However, significant mortality of 1st and 2nd instar nymphs originating from the treated eggs ($F_{10,142} = 18.50$, $P < 0.0001$) indicated a residual activity of fungal treatments on the leaf surface. All *B. bassiana* and *I. fumosorosea* isolates caused higher nymphal mortality, except for ESALQ1337 and ESALQ1409 (Fig. 4). These results indicated that newly hatched nymphs probably acquired conidia from the eggs soon after hatching or from leaf surface as secondary exposure.

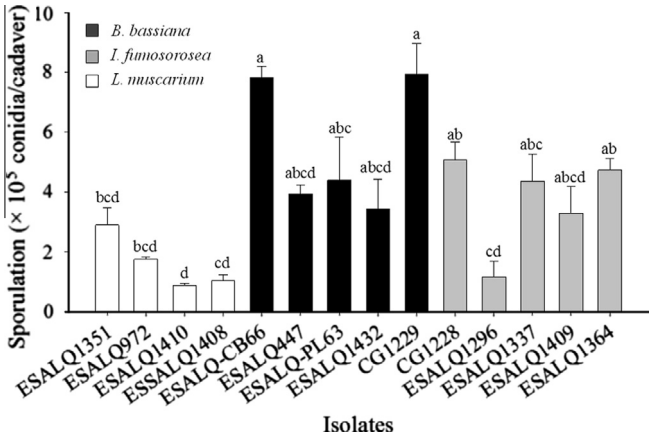

Fig. 3. Production of newly formed conidia by different *B. bassiana*, *I. fumosorosea* and *L. muscarium* isolates on mummified nymphal cadavers of *B. tabaci* biotype B. Error bars represent the standard error of the mean. Bars indicated by the same letters are not significantly different (Tukey's HSD, $P \leqslant 0.05$).

3.4. Virulence of best fungal isolates to adults

Adults of *B. tabaci* were susceptible to all *I. fumosorosea* isolates while only *B. bassiana* CG1229 caused > 50% mortality ($F_{9,106} = 5.73$, $P < 0.0001$) (Fig. 5). *I. fumosorosea* CG1228 caused the highest mortality of adults (93% mortality). This result indicated that adults effectively acquired conidia from the spray residue on the leaf surface. The sporulation on adults was affected by the fungal isolates ($F_{8,96} = 15.07$, $P < 0.0001$) with higher levels (50–89.5% sporulation) achieved by *I. fumosorosea* compared with the *B. bassiana* isolates (0.9–21%) (Fig. 5).

3.5. Conidial production using solid-state fermentation

B. bassiana and *I. fumosorosea* showed variability in spore production on rice ($F_{8,44} = 76.47$; $P < 0.0001$). Isolate CG1228 of *I.*

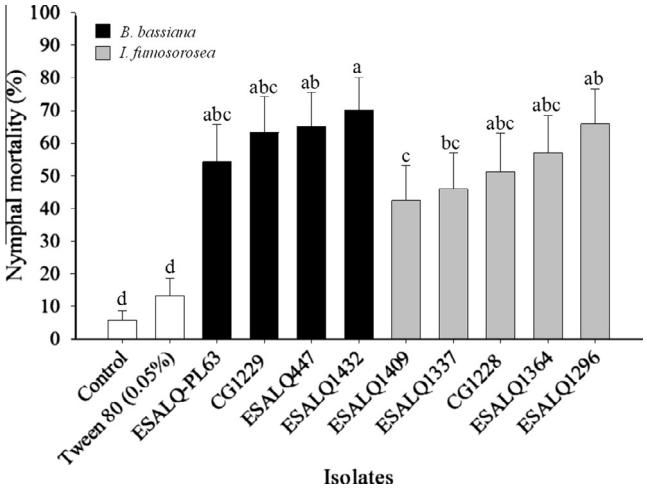

Fig. 4. Residual activity of *B. bassiana* and *I. fumosorosea* isolates on hatched nymphs by indirect uptake of conidia from treated leaves or eggs 11 d after *B. tabaci* biotype B eggs were exposed to 1674 conidia/mm^2 (=1 × 10^8 conidia/mL). Error bars represent the standard error of the mean. Bars indicated by the same letters are not significantly different (Tukey's HSD, $P \leqslant 0.05$).

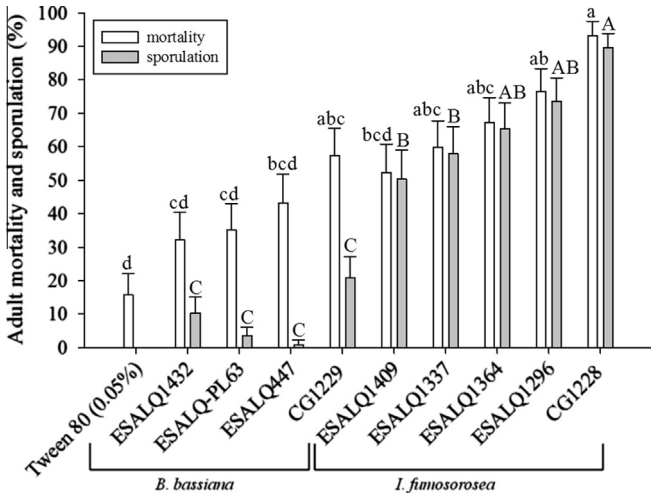

Fig. 5. Mortality and sporulation of *B. tabaci* biotype B adults 7 d after exposure to spray residue of different *B. bassiana* and *I. fumosorosea* isolates applied at 150 conidia/mm^2 (1×10^7 conidia/mL). Error bars represent the standard error of the mean. Bars indicated by the same letters, in lowercase for mortality and uppercase letters for sporulation, are not significantly different (Tukey's HSD test, $P \leqslant 0.05$).

fumosorosea provided the highest conidial yield, followed by isolate CG1229 of *B. bassiana* (Fig. 6). The other isolates had a production range of 0.9–2.3 $\times$ 10^9 viable conidia/g rice based on wet weight. The conidial viabilities for all these isolates were >95% in germination tests. Additional trials (data not shown, $n = 3$) showed high spore yields for CG1229 and CG1228 initially inoculated with 1×10^6 conidia/g parboiled rice and grown in plastic bags (250 g rice/bag). These isolates supported high yields of conidia after 7 d incubation at 26 °C (6.2 ± 1.3 $\times$ 10^{10} and 14.2 ± 1.7 $\times$ 10^{10} conidia/g of dry powder conidia with ~5% moisture content, respectively).

3.6. Surface hydrophobicity and spore-bound Pr1 activity

The majority of the *I. fumosorosea* and *B. bassiana* isolates showed high spore-bound Pr1 activity, except ESALQ1409 and ESALQ-PL63 that exhibited the lower enzyme activity ($F_{8,27} = 25.68$, $P < 0.0001$) (Table 3). The conidia of the fungal isolates had similar relative hydrophobicity ranging from 84.7 to 99.6% ($F_{8,18} = 1.76$; $P = 0.153$), though this parameter was not correlated with Pr1 activity or other virulence factors (Spearman's correlation: $-0.23 \leqslant r \leqslant 0.25$; $0.52 \leqslant P \leqslant 0.79$).

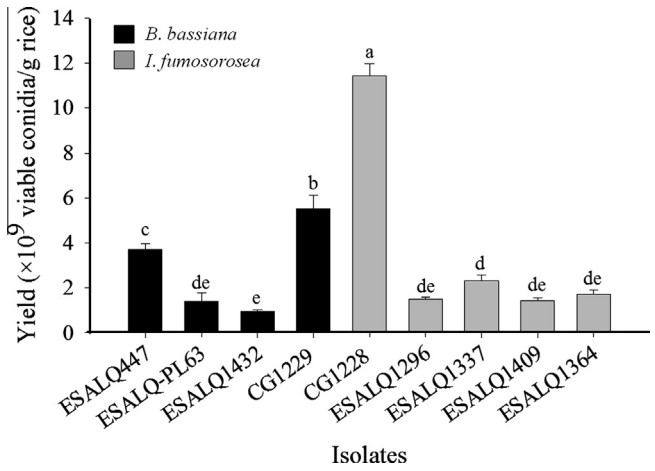

Fig. 6. Production of aerial conidia on rice (20 g/flask, wet weight) by different *B. bassiana* and *I. fumosorosea* isolates grown for 11 d on parboiled rice at 26 °C with a 14 h light regime. Error bars represent the standard error of the mean. Bars indicated by the same letters are not significantly different (Tukey's HSD, $P \leqslant 0.05$).

3.7. Cluster and principal component analysis

The PCA provided important information about the variability among fungal isolates and it was useful to find linear relationships between the observed variables, i.e. the performance of isolates in virulence and conidial production. Components 1, 2 and 3 explained 84.54% of the total variance and displayed eigenvalues substantially greater than one (Table 4). However, the biplot graph was drawn using only the two major components 1 and 2, which together explained ~70% of the information contained in the data set and comprised all the seven variables. Together components 1 and 2 consisted of significant variables at $P \leqslant 0.15$, according to Pearson's correlation method (Table 4). The contribution of each variable on each component axis in biplot graph was measured proportionally by the length of the arrows (Fig. 7A). The variables "adult mortality", "sporulated adults", "LT_{50} values", "mortality of hatched nymphs" and "conidial production on rice" were the most important for the first principal component, which, in turn, explained the maximal amount of total variance (41.32%). The second component explained 27% of total variance and comprised the variables "total nymph mortality" and "conidial production on nymphal cadavers". The biplot for individuals (Fig. 7B) pointed out isolates with high virulence to adults and nymphs as well as with high conidial yields. Three out of four *B. bassiana* isolates resulted in higher mortality and faster kill of hatched nymphs. Conversely, all isolates of *I. fumosorosea* and only *B. bassiana* (CG1229) were positively associated with other variables, indicating high virulence against nymphs and adults along with greater conidial production from rice and cadavers. *I. fumosorosea* CG1228 had a distinctive performance rather than the other isolates since it achieved the highest mortality and sporulation on adults and conidial production from rice. Nonetheless, both CG1229 and CG1228 showed high virulence against nymphs, as they caused high mortality rates with lower LT_{50} values.

The cluster analysis suggested four distinct groups with similar profiles (Fig. 8). The most closely related performances were identified by the shortest distances in the dendrogram. As a result, two single clusters comprised the isolates of *B. bassiana* (CG1229) and *I. fumosorosea* (CG1228). The other isolates of *B. bassiana* were grouped together in a seperate cluster from *I. fumosorosea* isolates.

Table 3
Relative hydrophobicity and spore-bound Pr1 enzyme activity for different isolates of *I. fumosorosea* and *B. bassiana* conidia produced on SDAY medium after 13 d of incubation at 26 °C with a 12 h photophase.

Fungus	Isolate	Relative hydrophobicity (%)	Spore-bound Pr1 (μmol/mL/min)[a]
I. fumosorosea	ESALQ 1296	84.7 ± 10.0[ns]	0.193 ± 0.002 a
	ESALQ 1337	88.8 ± 9.8	0.193 ± 0.002 a
	ESALQ 1364	96.1 ± 0.7	0.184 ± 0.002 a
	ESALQ 1409	94.4 ± 3.0	0.131 ± 0.012 b
	CG1228	85.0 ± 1.7	0.190 ± 0.002 a
B. bassiana	ESALQ 447	86.0 ± 1.7	0.190 ± 0.002 a
	ESALQ 1432	99.6 ± 0.1	0.196 ± 0.024 a
	ESALQ-PL63	99.6 ± 0.2	0.128 ± 0.010 b
	CG1229	98.9 ± 0.3	0.197 ± 0.002 a

Means (± SE) within a column followed by the same letter are not significantly different (Tukey's HSD, $P \leqslant 0.05$).
[a] Pr1 activity is expressed as μmol NA ml^{-1}min^{-1} released from succinyl-(Ala)2-Pro-Phe-NA.

Table 4
Principal components analysis with eigenvalues and variation explained for each principal component.

Component	Eigenvalue	Variation explained (%)	Cumulative variation (%)
1	2.89	41.32	41.32
2	1.89	26.99	68.32
3	1.14	16.22	84.54
4	0.82	11.68	96.21
5	0.22	3.20	99.42
6	0.04	0.56	99.98
7	0.002	0.02	100

Attribute loading for eigenvectors (rotated factor pattern) and correlations between variables and the three meaningful components retained[a]

Variable	Component		
	1	2	3
LT_{50}	−0.63 ($P = 0.07$)	–	–
Total nymph mortality	–	0.77 ($P = 0.01$)	–
Hatched nymph mortality	−0.52 ($P = 0.15$)	–	–
Conidial production on cadavers	–	0.91 ($P < 0.01$)	–
Conidial production on rice	0.66 ($P = 0.06$)	–	0.69 ($P = 0.04$)
Adult mortality	0.89 ($P < 0.01$)	–	–
Sporulated adults	0.86 ($P < 0.01$)	–	–

[a] Eigenvectors are presented only for components 1, 2, and 3. There were just three principal components retained which account for 84.54% of the total variance in the data set. Correlations (r, Pearson's method) between variables and the principal components with a significance threshold at $P \leqslant 0.15$ are shown. Since the components 1 and 2 comprised all the meaningful variables, the component 3 was no longer important to build the biplot graph.

4. Discussion

We evaluated different fungal attributes to identify virulent isolates against whitefly biotype B. Important criteria for selecting fungal pathogens for commercial development are high virulence against the target, potential for conidial production on artificial substrates and production of inoculum on insect cadavers. In our work, *I. fumosorosea* and *B. bassiana* isolates were virulent against whitefly nymphs, providing >70% mortality of 2nd instar nymphs with LT_{50} ⩽4 d. As previously stated by Wraight et al. (1998) and Lacey et al. (2008), the most virulent isolates to *B. tabaci* biotype B required 50–150 spores/mm^2 to cause 50% nymphal mortality, hence the majority of the Brazilian *I. fumosorosea* and *B. bassiana* isolates fell within this range. *L. muscarium* and other species of *Lecanicillium* have been used commercially as bioinsecticides against whiteflies throughout the world with some degree of success (Gindin et al., 2000; Faria and de Wraight, 2007; Goettel et al., 2008), but results indicated that the Brazilian *L. muscarium* isolates were comparatively less effective against *B. tabaci* biotype B. Cuthbertson et al. (2009) also verified a low efficacy (20–40% mortality) of a commercial isolate of *L. muscarium* (Mycotal®, Koppert Biological Systems Ltd., UK) against different lifestages of *B. tabaci* on poinsettia.

We noted that factors other than isolate, such as experimental method, host stage, environmental conditions and surfactant used, can affect the results of different bioassays. For example, Cabanillas and Jones (2009) prepared conidial suspensions with a 0.02% Silwet L-77® solution, whilst we suspended conidia in a 0.01% Tween 80 solution. Based on our previous studies, low concentrations of this organosilicone surfactant, as low as 0.01%, was toxic to 2nd instar whitefly nymphs inflicting up to 42% mortality after 7 d of exposure (Mascarin et al., unpublished). Thus, the type and concentration of chemical surfactants added in the conidial

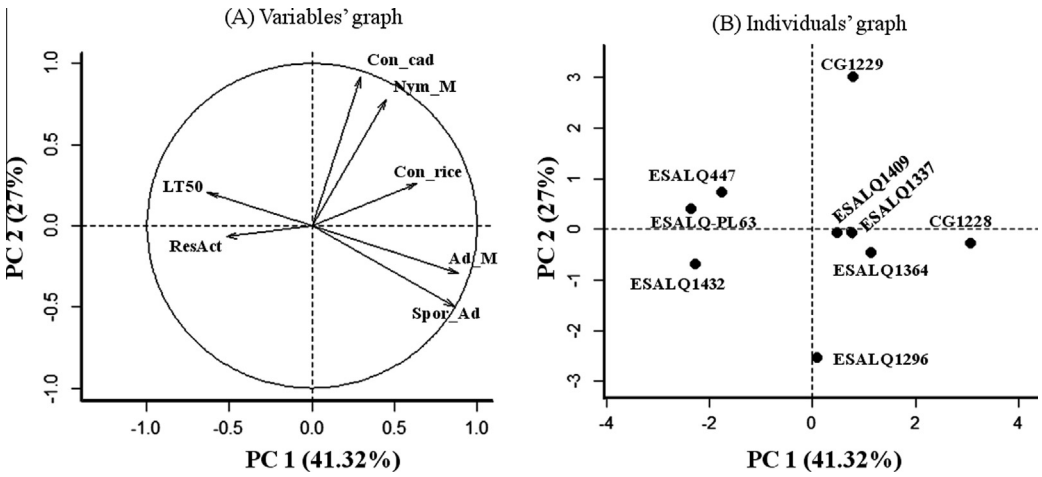

Fig. 7. Biplot pictures from PCA between components 1 and 2 which explained 68.32% of the total variance in the observed variables. (A) Variables' graph considering only active variables, in which the length of the arrows indicates the magnitude of its respective variable. (B) Individuals' graph showing the scores for the fungal isolates, according to the first two principal components. Component 1 comprises the variables "sporulated adults (Spor_Ad)", "adult mortality (Ad_M)", "hatched nymph mortality (ResAct)", "LT_{50} values (LT50)", and "conidial production on rice (Con_rice)", while component 2 is described by "total nymphal mortality (Nym_M)" and "conidial production on cadavers (Con_cad)".

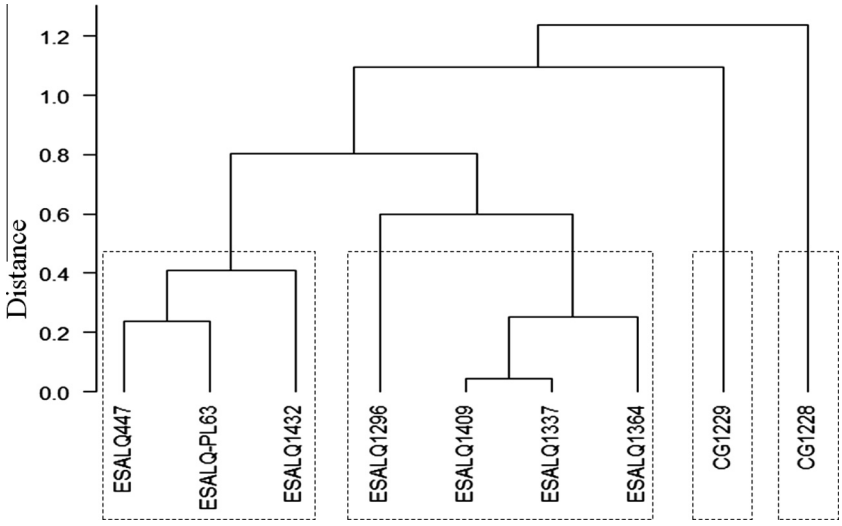

Fig. 8. Cluster dendrogram showing four distinct groups (surrounded by dashed rectangles) formed with nine isolates of *B. bassiana* and *I. fumosorosea* taking into account the meaningful response variables retained by PCA and using the unweighted pair-group method with arithmetic mean (UPGMA) to calculate the pairwise similarity distances.

suspensions can influence insect susceptibility, as confirmed by previous studies (Liu and Stansly, 2000; Srinivasan et al., 2008).

Several studies confirm the potential of *I. fumosorosea* and *B. bassiana* for the microbial control of whiteflies (Wraight et al., 1998, 2000; Vicentini et al., 2001; Ramos et al., 2000; Quesada-Moraga et al., 2006; Cabanillas and Jones, 2009). In Brazil, most registered mycopesticides are based on *M. anisopliae* sensu lato (Li et al., 2010), and no *Isaria* spp. product is currently available. Although *I. fumosorosea* has potential as a biological control agent for several insect pests (Zimmermann, 2008), it is rarely included in experiments in Brazil compared to *B. bassiana* and *M. anisopliae*. According to our results, the best candidates based on virulence to *B. tabaci* nymphs and adults were CG1228, ESALQ1364, ESALQ1409, ESALQ1296, and ESALQ1337 of *I. fumosorosea* and CG1229 of *B. bassiana*. The best isolates of *I. fumosorosea* and *B. bassiana* can be employed in inundative biocontrol approach against whiteflies, as these fungi are easily mass cultured on artificial solid substrates and are compatible with different types of formulation (Faria and Wraight, 2001; Jackson et al., 2010; Mascarin et al., 2010).

Estimates of in vivo sporulation varied among fungal species, which might affect horizontal transmission and disease development in the field. We observed proportionally greater sporulation responses from *I. fumosorosea* compared with *B. bassiana* isolates. However, *B. bassiana* produced equivalent or higher quantities of conidia whereas only low spore production was achieved by *L. muscarium* isolates. It has been shown that *I. fumosorosea* can incite epizootics in whitefly populations under suitable weather conditions (Lacey et al., 2008). This capability represents an advantage over *B. bassiana*, but in practice the impact of transmission of *Isaria* on the host population in the field will be contingent on prolonged periods of very cloudy and wet weather. Conidiation on whitefly cadavers is very dependent on high relative humidity but also varies with temperature, fungal isolate, host species, host stage and incubation time (Sosa-Gómez and Alves, 2000).

We observed low mortality of whitefly eggs treated with fungi, confirming earlier reports that eggs have low susceptibility to entomopathogenic fungi (Negasi et al., 1998; Lacey et al., 1999; Gindin et al., 2000; Ramos et al., 2000). However, our results demonstrated that conidia remained active and infected a substantial proportion of emerging nymphs, suggesting that application might be targeted against this stage. Fransen et al. (1987) also noted a better efficacy of *Aschersonia aleyrodis* to newly emerged nymphs from treated eggs.

The production of aerial conidia of fungal entomopathogens cultured on solid substrates also varied among species and strains. In Brazil, rice is most commonly used in solid-state fermentation of fungal entomopathogens (Li et al., 2010). The greatest conidial yield was attained by *I. fumosorosea* CG1228 cultured on parboiled rice. In contrast, Mascarin et al. (2010) reported the maximal yield of *I. fumosorosea* ESALQ1296 as 1.1×10^9 viable conidia/g of whole rice in a biphasic fermentation process. Parboiled rice appears to be highly conducive to producing higher yields of conidia in solid-state fermentation due to its better nutritional and physical properties. *B. bassiana* CG1229 was the second-rank fungus in terms of conidial production on rice.

Several studies show that enzymes and hydrophobicity correlate with fungal virulence (Shah et al., 2005, 2007). We did not find significant positive or negative correlation of enzyme Pr1 and hydrophobicity with the virulence factors of either *B. bassiana* or *I. fumosorosea.* This outcome might suggest that other virulence factors are involved in the infection process not addressed here.

Principal component analysis (PCA) and cluster analysis suggested that median lethal time (LT_{50}), total nymph mortality, hatched nymph mortality, in vitro conidial production, in vivo sporulation on nymphal cadavers, adult mortality, and in vivo sporulation response amongst adult whiteflies were significant variables for virulence screening assays. In our investigation, the other variables such as egg mortality and in vivo sporulation on eggs along with relative hydrophobicity and Pr1 enzyme activity of conidia did not affect virulence of isolates against whitefly. The isolate CG1228 of *I. fumosorosea* can be considered a promising candidate for inundative biocontrol approach against *B. tabaci* biotype B based on our results, and will be selected for commercial development, while *B. bassiana* CG1229 was also promising.

Given the elevated pest status of *B. tabaci* biotype B in Brazil, the development of virulent entomopathogenic fungus is highly desirable. In crops such as beans and tomatoes, where *Bemisia* is a vector for geminivirus, population must be kept below action thresholds (Faria and Wraight, 2001). Therefore, further studies are underway to identify effective formulation techniques to optimize the efficacy and persistence of fungal isolates under field conditions, for adoption in the integrated whitefly management.

Acknowledgments

We are very grateful to Rayan C.J. Vital, Rodrigo A. Silva, Edmar M. Cardoso and Edson D. D. Jacinto for their technical assistance with several experiments. Special thanks to Márcio V.C.B. Cortes for his advice on the enzymatic assays, to Dr. Paulo R. Queiroz da Silva for whitefly identification, and Christopher Dunlap (USDA-Peoria) for molecular identification of our fungal isolates. We also express our sincere gratitude to Mark Jackson (USDA-Peoria), Steven Arthurs (UF-Apopka) and Marcos Faria (EMBRAPA) for their critical and valuable feedback on earlier drafts. This work was partially supported by the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), CNPq (project 563233/2010-9) and FAPESP (2010/52342-4). The mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply the recommendation or endorsement by EMBRAPA or by ESALQ-USP.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2013.05.001.

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