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Selection of *Beauveria bassiana* isolates for control of the whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* on the basis of their virulence, thermal requirements, and toxicogenic activity

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Abstract

As part of a 3-fold approach to select potential mycoinsecticides for whitefly control, we evaluated infectivity, thermal requirements, and toxicogenic activity of the entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Clavicipitaceae) under laboratory conditions. Twenty-five native *B. bassiana* isolates and a commercially available mycoinsecticide (based on *B. bassiana*) were evaluated for virulence to fourth instar nymphs of sweetpotato whitefly, *Bemisia tabaci*, and greenhouse whitefly, *Trialeurodes vaporariorum*, at a concentration of 1×10^7 conidia/ml. All isolates were pathogenic for both whitefly species, whereas mortality rates varied from 3 to 85%. A second series of bioassays was conducted on 10 selected isolates using four 10-fold concentrations ranging from 1×10^5 to 1×10^8 conidia/ml. Median lethal concentrations (LC_{50}) of the four most virulent isolates varied from 1.1×10^5 to 6.2×10^6 conidia/ml and average survival time (AST) of treated nymphs from 5.9 to 7.4 days. *T. vaporariorum* were significantly more susceptible to all *B. bassiana* isolates than *B. tabaci*. The thermal biology of the eight most virulent isolates to both whitefly species was investigated at six temperatures (10–35 °C). The colony radial growth rate was estimated from the slope of the linear regression of colony radius on time and data were then fitted to a modified generalized β function that accounted for 90.5–99.3% of the data variance. Optimum temperatures for extension rate ranged from 23.1 to 27.1 °C, whereas maximum temperatures for fungal growth varied from 31.8 to 36.6 °C. On the basis of their virulence and thermal requirements, three isolates showed promise as candidates for whitefly management in Mediterranean greenhouses. Whilst in vitro production of macromolecular compounds toxic to *Galleria mellonella* larvae was not a requisite for virulence, ASTs of larvae injected with Sephadex G-25 fractions from candidate isolates ranged from 1.4 to 3.7 days compared with 5–6 days for non-toxic G-25 fractions. In addition, proteinase K treatment significantly reduced their toxic activity suggesting that they were proteins and revealing the potential of these isolates to be further improved through biotechnology to kill the pest more quickly.

Keywords: *Beauveria bassiana*; *Bemisia tabaci*; *Trialeurodes vaporariorum*; Sweetpotato whitefly; Greenhouse whitefly; Macromolecular toxins; Entomopathogenic fungi; Insecticidal proteins; Mycoinsecticides

1. Introduction

The whitefly, *Bemisia tabaci* (Gennadius), is a major pest of indoor and outdoor crops in warm climates worldwide. It causes direct damage by feeding on leaves and also indirect damage by promoting growth of black sooty mold on their honeydew secretions and by vectoring economically important plant viruses (Faria and Wraight, 2001; Osborne and Landa, 1992; Varma and Malathi, 2003). Although *B. tabaci* is a problem in Mediterranean countries, the highly polyphagous greenhouse whitefly, *Trialeurodes vaporariorum* Westwood, still remains among the major insect pests of many greenhouse crops in Europe.

Control of *B. tabaci* and *T. vaporariorum* is primarily accomplished through the use of conventional insecticides.

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Chemical control has resulted in the development of resistant *B. tabaci* and *T. vaporariorum* populations, and its negative environmental impact has encouraged the development of alternative pest management strategies, in which microbial control may play an important role (Faria and Wraight, 2001). Whiteflies feed by piercing the tissues of plants and sucking sap directly from the vascular bundles. Consequently, entomopathogenic fungi, which are the only insect pathogens infecting their hosts by direct penetration of the cuticle, show promise for their control (Faria and Wraight, 2001). Laboratory and field studies have revealed *Beauveria bassiana* (Bals.) Vuill. to be an excellent pathogen of *B. tabaci* and *T. vaporariorum* when applied directly as a concentrated conidial suspension (Carruthers et al., 1993; Eyal et al., 1994; Fargues et al., 2003; Garza and Arredondo, 1993; Poprawski and Jones, 2000; Wraight et al., 1998; Wright, 1992), and together with *Paecilomyces fumosoroseus* (Wize) and *Lecanicillium* sp., have been registered as microbial control agents for whitefly management (Bolckmans et al., 1995; Faria and Wraight, 2001; Ravensberg et al., 1990; Wright, 1992). Nevertheless, acceptance of these products has been limited probably due to the general perception among farmers that, compared with conventional chemical products, they are not as fast acting and lose their effectiveness more rapidly (St. Leger and Screen, 2001). Accordingly, for controlling whiteflies it is necessary to select fungal isolates that combine the best characteristics for killing the target insects as (i) high virulence against target organisms, (ii) the ability to persist and infect in the environment in which the pest is occurring, and (iii) the potential for being biotechnologically improved to kill the insect host more quickly.

The first step in developing a microbial control program is laboratory evaluation of the effectiveness of potential microbial agents. Yeo et al. (2003) have proposed a ''biorational approach'' for selecting isolates of entomopathogenic fungi for pest control. It is based not only on intrinsic virulence of fungal isolates to the target host revealed by laboratory bioassays, but also on their ability to operate over the range of abiotic conditions that they would encounter in the agro-ecosystem. The general belief that moist ambient conditions are essential for effective use of fungi in microbial control does not necessarily hold true in all situations. Some studies have revealed that the ability of entomopathogenic fungi to germinate and infect the host under conditions of low ambient humidity is attributed to sufficient moisture within microhabitats (Fargues et al., 2003; Inglis et al., 2001; Wraight et al., 2000). In contrast, ambient temperature influences the rate of infection and time to death of insects treated with entomopathogenic anamorphic fungi and is a key factor influencing efficacy of these biocontrol agents (Inglis et al., 2001). Therefore, it is important to match the thermal tolerance of a prospective fungal isolate to the climatic conditions expected at the targeted environment (Faria and Wraight, 2001).

Unfortunately, a major hindrance to the development of entomopathogenic fungi as mycoinsecticides has been that, compared with chemical insecticides, they require a longer time after application for insect control (5–10 days), during which the infected insects can cause serious damage to the crops (St. Leger and Screen, 2001). The most economically important virus vectored by whiteflies is the tomato yellow leaf curl virus (TYLCV) that is transmitted by adults, and any control strategy has to prevent nymphs from reaching the adult stage. Thus, the above hindrance may limit the effectiveness of fungal treatments for whitefly control because part of the nymphal population may reach the adult stage before succumbing to fungal infection. For that, a key aim of most recent work has been to improve the effectiveness of mycoinsecticides, which may be obtained by optimizing production, stability and application of the inoculum, and through genetic modification (Fang et al., 2005; St. Leger and Robert, 1997; St. Leger and Screen, 2001).

We suggest that it would be of great interest to add among our strain selection criteria the potential of isolates to be biotechnologically improved to kill the insect host more quickly. Different isolates from the same fungal species (i.e., *B. bassiana*) may have different means to kill the insect host with one of them based on the production of toxins as virulence factors (Kershaw et al., 1999; Roberts, 1981; Vey et al., 2001). Among them there are well-characterized low molecular weight insecticidal cyclic peptides (Kachatourians, 1996; Roberts, 1981; Vey et al., 2001) and poorly studied macromolecular insecticidal proteins some of which have been reported as virulence factors (Fuguet et al., 2004; Fuguet and Vey, 2004; Mazet et al., 1994; Mazet and Vey, 1995; Quesada-Moraga and Vey, 2003, 2004). Consequently, to make significant progress in the understanding of fungal pathogenesis, it is necessary to analyze in more detail the in vitro secretion of large molecular weight compounds, and to establish if such molecules are also secreted in vivo and whether they play a significant role in the pathogenesis of fungal infections. In case they do, they are key components amenable to improvement via biotechnology (Hegedus and Khachatourians, 1995; St. Leger and Screen, 2001).

As part of this approach, the aims of our studies were 3-fold: (i) to identify native isolates of *B. bassiana* possessing good virulence against *B. tabaci* and *T. vaporariorum*, (ii) to study the thermal biology of candidate isolates, and (iii) to evaluate the in vitro production of active macromolecules among candidate isolates. The information from objectives (i) and (ii) could be used to inform the selection of isolates for whitefly biological control, whereas objective (iii) could reveal the potential of candidate isolates to be further improved through biotechnology to kill the pest more quickly.

2. Materials and methods

2.1. Insects

The *B. tabaci* population originated from the stock colony of La Mayora C.S.I.C. Research Station, Malaga,

Spain. The *T. vaporariorum* population was established from a colony collected from geranium plants (*Pelargonium* sp.) from Cordoba. Whiteflies were mass-reared in screened cages ($40 \times 40 \times 30$ cm) containing young green melon plants (*Cucumis melo* L., cv. Galia) at 26 ± 2 °C, 65% RH with a 14:10 (L:D) photoperiod. To produce plants with heavy and homogeneous nymphal population levels for virulence experiments, melon plants with four leaves were individually placed into cylindrical cages (diameter: 14 cm, height: 24 cm) with organdysleeve (300-μm mesh) and infested by introducing 100 young adult whiteflies (sex ratio ≈ 1:1). Whitefly adults were allowed to lay eggs for 2 days, resulting in at least 50–100 eggs/leaf.

Galleria mellonella (L.) larvae were reared in a room at 26 ± 2 °C and 12:12 (L:D). Larvae were confined to glass jars and maintained on an artificial diet composed of a mixture of honey, 250 g; glycerol, 220 ml; wheat meal, 340 g; yeast powder, 100 g; pure beeswax, 50 g; nipagin, 1.75 g.

2.2. Fungal isolates

Isolates of *B. bassiana* selected for screening originated from diverse sites in southern Spain (Table 1); there were six isolates from orthopteran and lepidopteran hosts and 20 from soil samples from different habitats. Slant monoconidial cultures of strains were grown on malt agar (MA) at 25 °C in darkness and then stored at 4 °C. The ATCC 74040 *B. bassiana* isolate from the product Naturalis L (Troy Biosciences, AZ, USA) (Wright, 1992) was also included as a reference isolate.

2.3. Fungal preparations

To produce inocula for experiments, slant cultures of the selected strains from the collection were subcultured by mixed conidial transfer to MA petri plates that were always placed for 15 days at 25 °C in darkness. Petri plates were sealed with Parafilm and freshly collected conidia from 15-day-old cultures were used for every experiment and each replicate run. Conidial suspensions were prepared by scraping conidia from petri plates into an aqueous solution of 0.002% Tween 80. The conidial suspension was filtered through several layers of cheesecloth to remove mycelial mats. Viability of conidia was assessed before preparation of suspensions by germinating tests in liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium. In all experiments, germination rates were higher than 95% after 24 h at 25 °C. The concentration of conidia in the final suspension was determined using a hemocytometer. The conidial suspension used for the first series of bioassays was adjusted by diluting conidia with 0.002% Tween 80 to a final concentration of 10^7 conidia/ml. In the second series of virulence bioassays, we used four concentrations of conidia of 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml. In all cases, replicate dilution series for inoculation of replicate leaf disks were prepared.

2.4. Bioassay protocol

In the first series of bioassays, fungal virulence was assessed in bioassays with *B. tabaci*. Representative isolates

Table 1
Identity of *B. bassiana* isolates from the culture collection at C.R.A.F. Department of the University of Cordoba assayed against *B. tabaci* and *T. vaporariorum*

Isolate	Insect host or substrate (habitat)	Site and date of origin
EABb 90/2-Dm	*Dociostaurus maroccanus* (Orthoptera: Acrididae)	Badajoz (Spain), 1990
EABb 90/4-Cb	*Chortipus bicolor* (Orthoptera: Acrididae)	Badajoz (Spain), 1990
EABb 91/6-Ci	*Calliptamus* sp. (Orthoptera: Acrididae)	Badajoz (Spain), 1991
EABb 91/7-Dm	*Dociostaurus maroccanus* (Orthoptera: Acrididae)	Badajoz (Spain), 1991
EABb 93/14-Tp	*Thaumetopoea pityocampa* (Lepidoptera: Notodontidae)	Córdoba (Spain), 1993
EABb 00/23-Su	Soil (pasture)	Tenerife (Spain), 2000
EABb 00/29-Su	Soil (citrus orchard)	Córdoba (Spain), 2000
EABb 01/11-Su	Soil (forest)	Córdoba (Spain), 2001
EABb 01/12-Su	Soil (non-cultivated)	Sevilla (Spain), 2001
EABb 01/14-Su	Soil (wheat)	Sevilla (Spain), 2001
EABb 01/15-Su	Soil (non-cultivated)	Almería (Spain), 2001
EABb 01/17-Su	Soil (vegetables)	Almería (Spain), 2001
EABb 01/36-Su	Soil (pasture)	Málaga (Spain), 2001
EABb 01/39-Su	Soil (almond tree orchard)	Málaga (Spain), 2001
EABb 01/53-Su	Soil (olive tree orchard)	Jaén (Spain), 2001
EABb 01/77-Su	Soil (vegetables)	Granada (Spain), 2001
EABb 01/81-Su	Soil (forest)	Sevilla (Spain), 2001
EABb 01/85-Su	Soil (forest)	Huelva (Spain), 2001
EABb 01/105-Su	Soil (cotton)	Sevilla (Spain), 2001
EABb 01/110-Su	Soil (oak)	Sevilla (Spain), 2001
EABb 01/112-Su	Soil (wheat)	Sevilla (Spain), 2001
EABb 01/143-Su	Bare soil	Sevilla (Spain), 2001
EABb 01/145-Su	Soil (olive tree orchard)	Sevilla (Spain), 2001
EABb 01/147-Su	Bare soil	Sevilla (Spain), 2001
EABb 01/157-Su	Soil (sunflower)	Cádiz (Spain), 2001

showing low, medium, and high activity against *B. tabaci* were then assessed against *T. vaporariorum*. Newly molted fourth instar *B. tabaci* and *T. vaporariorum* nymphs were used for all screening bioassay procedures. Individual melon leaf disks (30-mm diameter) with approximately 20–40 nymphs were used immediately upon excision. These leaf pieces bearing nymphs were immersed in 20 ml of a conidial suspension for 10 s, one disk per suspension and control leaves were immersed in 0.002% Tween 80 for the same length of time. To prevent development of saprophytic fungi, treated leaves were placed on filter paper for 20–30 min to remove excess moisture. The leaves were then placed in 5% water–agar 90-mm diameter petri plates, which were sealed with Parafilm and incubated in a growth chamber at 26 ± 2 °C and 14:10 (L:D) photoperiod. Relative humidity was maintained close to 100% in each petri plate. For aeration purposes, each plate was opened daily for 25–30 min. This procedure was necessary to minimize development of saprophytic fungi on whitefly honeydew. The number of insects per leaf sector was counted prior to inoculation. The total number of dead whiteflies (nymphs desiccated and/or discolored and mycosed individuals) and infected whiteflies (as defined by symptomatic larvae-pink/red coloration, fungal outgrowth or conidiation on the cadavers) were recorded daily for 8 days. Both whiteflies were tested using the same conidial stock suspensions. For each whitefly species, all strains were assayed at one time, using randomized groups of insects from a single batch. Four replicate infested leaf pieces were used for each isolate, and the whole experiment was repeated with a new batch of insects and new conidial suspensions. Mortality data were analyzed using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test was used to compare means. Statistical analyses were performed using SPSS 8.0 for Windows (SPSS, 1997).

In the second series of virulence bioassays, isolates causing the greatest mortality against *B. tabaci* and *T. vaporariorum* were selected for further virulence studies against both species in separate bioassays. The basic measures of virulence included the estimated concentration required to kill 50% of the test insects (LC_{50} expressed as conidia/ml) and the average survival time (AST) in days of the treated insects. Each individual assay of a selected fungal strain comprised four concentrations at 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml, each with four replicate leaf pieces containing 20–24 whitefly nymphs and four control leaf pieces treated with a solution of 0.002% Tween 80. The whole experiment was repeated with a new batch of insects and new conidial suspensions. The total number of dead whiteflies and infected whiteflies were recorded daily for 8 days. Median lethal concentrations (LC_{50}) were estimated by probit analysis (Finney, 1971) using SPSS 8.0 for Windows (SPSS, 1997). Concentration responses were corrected for control mortality by the program. Tests of parallelism of probit regression lines for all isolates assayed against each whitefly species and for pairs for those isolates assayed against both whitefly species were made with SPSS 8.0 for Windows by using χ^2 goodness-of-fit tests. Relative median potencies and their 95% confidence intervals were calculated for different treatments when their slopes did not differ significantly (Finney, 1971). The cumulative mortality response across the assessment period was analyzed with Kaplan–Meier survival analysis by using the SPSS program. Correlation (Pearson) between AST and log LC_{50} values for both whitefly species was also performed using the above program.

2.5. Effect of temperature on in vitro radial colony growth of fungi

For each selected isolate, circular plugs (5-mm diameter) were cut from non-sporulating mycelia of 7-day-old culture dishes using a cork-borer and a single plug was placed upside down in the center of a new dish of MA medium. Dishes were sealed with Parafilm and incubated in the dark in separate incubators at 10, 15, 20, 25, 30, and 35 ± 1 °C. Five replicate dishes were prepared for each isolate and temperature combination. Surface radial growth was recorded daily using two cardinal diameters previously drawn on the bottom of the dish. The experiment was run for 10 days or until the fungal colony had covered the petri dish.

Radial growth data were fitted by regression analysis using SPSS 8.0 for Windows. Because radial measurements (from the 2nd to the 10th day) fitted a linear model ($y = vt + b$), the linear regression slopes (v), which indicated the growth rates (velocity in mm per day), were used as the main parameter to evaluate the influence of temperature on fungal growth (Davidson et al., 2003; Fargues et al., 1992; Ouedraogo et al., 1997; Yeo et al., 2003). For that, a generalized β function, modified according to Bassanezi et al. (1998), was fitted to the average growth rates under different temperatures. The generalized β function is given by:

$$Y(T) = TY_{\mathrm{opt}}[(T - T_{\min})/(T_{\mathrm{opt}} - T_{\min})] \exp[TB_3(T_{\mathrm{opt}} - T_{\min})/(T_{\max} - T_{\mathrm{opt}})][(T_{\max} - T)/(T_{\max} - T_{\mathrm{opt}})] \times \exp TB_3,$$

where $Y(T)$ is the fungal growth in mm per day (dependent variable) and T is the incubation temperature (independent variable). $T_{\min}$, $T_{\max}$, and T_{opt} are, respectively, the lowest, the highest, and the optimal temperature for fungal growth. TY_{opt} is the fungal growth at the optimal temperature T_{opt}. TB_3 is the shape parameter that influences the temperature range around T_{opt} in which the curve stays near to TY_{opt}; for low values, for example 0.1, a broad temperature range exits while for high values, for instance 3.0, the curve sharply declines when temperature differs only slightly from T_{opt} (Bassanezi et al., 1998). On the basis of our preliminary experiments, $T_{\min}$ was fixed at 5 °C.

TY_{opt}, T_{opt}, $T_{\max}$, and TB_3 values were estimated by the method of Levenberg–Marquardt (Bates and Watts, 1988). The validation of the model was based on the coefficient of

determination r^2 values and on the signification of the standard errors of the parameters (Campbell and Madden, 1990). A separate β function analysis was conducted on the data from each of the five replicates (one from each temperature), which finally yielded five replicates of each parameter. Multivariate analysis of variance (MANOVA) was used to compare parameters. When the model was shown to be significantly different for different isolates, one-way analysis of variance (ANOVA) was performed for each parameter (Campbell and Madden, 1990). Analyses of variance were followed by comparison of means using least significant difference (LSD). Statistical analyses were performed by using SPSS 8.0 for Windows (SPSS, 1997).

2.6. Production of insecticidal macromolecules by fungal isolates in Adamek's medium

Conidial suspensions of selected isolates were prepared as described previously. To prepare a primary culture, 1 ml of a suspension of conidia (adjusted to 1×10^7 conidia/ml) was inoculated into 25 ml of Adamek's liquid medium (glucose, 40 g; yeast extract; 40 g; corn steep liquor, 30 g; 1000 ml distilled water) in 100 ml Erlenmeyer flasks and cultured at 25 °C on a rotatory shaker (OVAN Multimix) at 110 rpm for 4 days. To inoculate secondary cultures for large-scale growth of the isolates, 2 ml of the primary culture was transferred into 250 ml of the same medium in each of 6 one-liter Erlenmeyer flasks for each isolate, and cultured on the above shaker at 25 °C and 110 rpm for 7 days before removing the mycelial material by filtration through Whatman No. 3 filter paper. Proteins from the filtrates of each isolate, precipitated with 90% of saturation of ammonium sulfate, were collected by centrifugation at 10,000*g* for 30 min. The precipitate was desalted by gel filtration through a Sephadex G-25 (Pharmacia) column (2.5×30 cm) in 50 mM Tris/HCl buffer, pH 8.0. Seven-ml fractions were collected, and those corresponding to the excluded peak were pooled and concentrated before being injected. Total protein concentration was determined with the Bio-Rad Protein Assay based on the method of Bradford (1976). Bovine serum albumin was used as the standard.

The desalted fractions were injected into fourth instar *G. mellonella* larvae through the intersegmental membrane between second and third abdominal segments after sterilization through a 0.2 μm Dynagard filter unit, using a Burkard microinjector. Control insects were injected with 8 μl of the Sephadex G-25 desalted fraction of the Adamek's liquid medium with the same total protein concentration. The G-25 desalted fraction of each isolate was injected to 40 larvae, four replicates of 10 larvae, and the whole experiment repeated with new batches of insects and new fungal cultures extracted to produce new culture filtrates. The bioassay was monitored daily for 7 days. Dead insects were dissected under a binocular microscope (Nikon SMZ 800) to identify signs of activity of the injected molecules on insect tissues. Observations on the presence of any chromatic changes or morphological alterations on tracheae, air sacs, digestive track, fat body, muscle, and cuticle were made. Samples of tissues showing signs of insecticidal activity were also examined with a Leica Leitz DMRB optic microscope. To study the effect of protease treatment on the insecticidal activity of the proteins in the G-25 desalted fractions, G-25 fractions containing 1 mg of protein/ml were incubated for 2 h at 55 °C with a solution of Proteinase K (Sigma, Spain) and then injected into fourth instar *G. mellonella* larvae as previously described. The G-25 desalted fractions of each isolate before and after protease treatment were injected as described above using the same experimental design. Mortality data were analyzed with one-way analysis of variance (ANOVA) and the least significant difference (LSD) test was used to compare means. The cumulative mortality response across the assessment period was analyzed with Kaplan–Meier survival analysis. Statistical analyses were performed using SPSS 8.0 for Windows (SPSS, 1997).

3. Results

3.1. Virulence of B. bassiana on nymphs of B. tabaci and T. vaporariorum

Virulence of 25 strains of *B. bassiana* isolated from various insects and soils from different regions of southern Spain and the reference ATCC 74040 isolate to fourth instar *B. tabaci* nymphs was compared (Fig. 1). There was a significant effect of the fungal treatment ($F_{26,189} = 166$, $P < 0.001$ for 1×10^7 conidia/ml), with mortality of nymphs varying from 3 to 78%, compared with mortality of uninoculated whiteflies, which ranged between 1.5 and 2.5% (Fig. 1). Three groupings amongst the isolates could be distinguished: a first group of least virulent isolates (<10% mortality), a second group was intermediate in virulence (10–50% mortality) and a third group was highly virulent (>50% mortality). Four from the 25 assayed isolates caused greater mortality than reference ATCC 74040 isolate, which was included in the intermediate group (33.2 ± 6.4% mortality). The most virulent isolates were EABb 01/110-Su, EABb 93/14-Tp, EABb 90/4-Cb, and EABb 01/12-Su, which caused 77.7, 68.0, 66.6, and 56.7% mortality, respectively. All *B. bassiana* isolates produced an unidentified pigment within the host hemocoel that colored most infected nymphs various intensities of red making infected individuals readily identifiable by eye against the dark green background of the melon leaf. Sometimes, postmortem hyphal growth and sporulation of *B. bassiana* rapidly covered the dead host and extended several mm onto the surrounding leaf surface.

Isolates EABb 01/39-Su from the least virulent group, EABb 01/147-Su from the intermediate group and EABb 90/2-Dm, EABb 93/14-Tp, and EABb 01/110-Su from the highly virulent group were also assessed against fourth instar nymphs of *T. vaporariorum* (Fig. 1). There was a

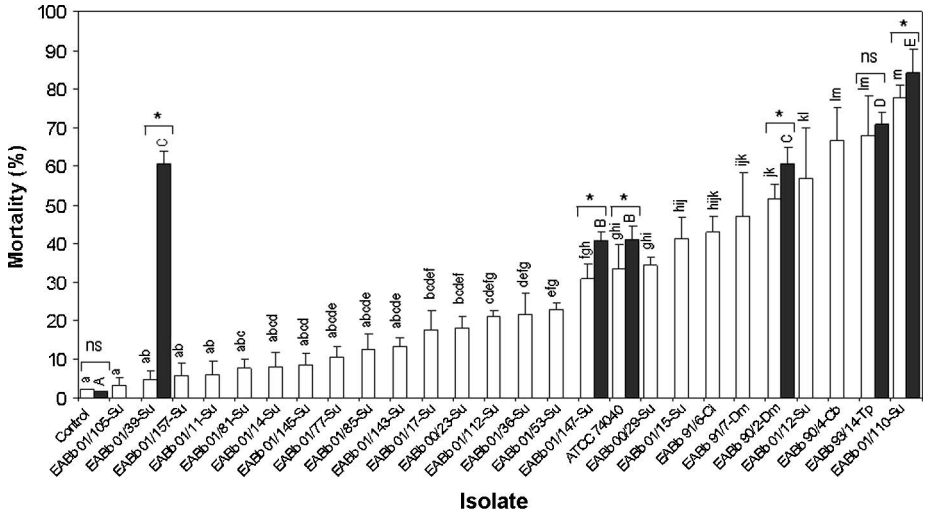

Fig. 1. Percentage mortality (mean ± SE) of fourth instar nymphs of *B. tabaci* (white bars and lowercase) and of *T. vaporariorum* (black bars and capital) 8 days after inoculation by conidial suspensions of several *B. bassiana* strains (1×10^7 conidia/ml). For each whitefly species, bars with the same letter are not significantly different ($P < 0.05$) according to the Tukey (HSD) test. Asterisks indicates the existence of significant differences among strains inoculated to both whitefly species according to the Tukey (HSD) test ($P < 0.05$).

significant effect of the fungal treatment ($F_{6,49} = 89.8$, $P < 0.001$ for 1×10^7 conidia/ml), with mortality of nymphs recorded on the 8th day ranging between 40 and 85%, compared to 1–2% mortality of uninoculated whiteflies (Fig. 1). Nymphs of *T. vaporariorum* inoculated with isolates from the highly virulent group suffered mortalities >50%; the most virulent isolates were EABb 01/110-Su and EABb 93/14-Tp causing 84.3 and 70.8% mortality, respectively. Conversely, isolate EABb 01/39-Su, which exhibited less than 5% mortality on *B. tabaci*, caused 60.7% mortality on *T. vaporariorum*. All isolates, except EABb 93/14-Tp ($F_{1,14} = 0.47$, $P = 0.5$), caused significantly higher mortality to *T. vaporariorum* than to *B. tabaci* at the concentration of 1×10^7 conidia/ml ($F_{1,14} = 8.00$, $P = 0.0134$; $F_{1,14} = 21.86$, $P = 0.0004$; $F_{1,14} = 41.50$, $P < 0.0001$; $F_{1,14} = 42.00$, $P < 0.0001$; $F_{1,14} = 2179$, $P < 0.0001$ for isolates EABb 01/110-Su, EABb 90/2-Dm, EABb 01/147-Su, ATCC 74040, and EABb 01/39-Su, respectively) (Fig. 1). No significant differences were observed between *B. tabaci* and *T. vaporariorum* uninoculated whiteflies ($F_{1,14} = 1.36$, $P = 0.26$). Signs of fungal infection in *T. vaporariorum* were similar to those described above for *B. tabaci*.

In the second series of virulence bioassays, we selected the most virulent isolates together with isolate EABb 01/147-Su from the intermediate group and EABb 01/39-Su from the least virulent group for comparison. The concentration–mortality response regression analysis for each isolate was calculated by assaying four concentrations (1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml) against fourth instar nymphs of *B. tabaci* (Table 2). Regression coefficients from all assays were low, with mean values for the individual isolates varying from 0.21 to 0.55. All χ^2 values were not significant ($\alpha = 0.05$) indicating good fit of the regression lines. The maximum mortality achieved at the higher concentration (1×10^8 conidia/ml) ranged between 60.0% (isolate EABb 01/147-Su) and 80.3% (isolate EABb 01/110-Su). LC_{50} values varied from 6.53×10^5 conidia/ml (isolate EABb 01/110-Su) to 3.50×10^8 conidia/ml (isolate ATCC 74040). Further, a test for parallelism showed that the data could be represented by nine parallel log concentration-probit lines ($\chi^2 = 13.1$, with 8 *df*) with a common slope (0.4 ± 0.05, mean ± SE). The relative potencies and the 95% fiducial limits of the treatment with isolates EABb 01/110-Su, EABb 93/14-Tp, EABb 90/4-Cb, EABb 01/12-Su, EABb 91/6-Ci, EABb 91/7-Dm, EABb 90/2-Dm, and EABb 01/147-Su with respect to the treatment with reference isolate ATCC 74040 were 119 (11–454), 38 (5–793), 37 (5–781), 29 (4–515), 10 (2–93), 6 (1–56), 4 (1–29), and 0.6 (0.1–2.0), respectively, indicating that all isolates, except EABb 01/147, were more virulent to *B. tabaci* than the reference ATCC 74040 isolate.

Regression coefficients from assays against *T. vaporariorum* were also low, with mean values for the individual isolates varying from 0.31 to 0.51. All χ^2 values were not significant ($\alpha = 0.05$) indicating good fit of these regression lines. The maximum mortality achieved at the higher concentration (1×10^8 conidia/ml) ranged between 61% (isolate EABb 01/147-Su) and 89.4% (isolate EABb 01/110-Su). LC_{50} values varied from 1.61×10^5 conidia/ml (isolate EABb 01/110-Su) to 3.06×10^7 conidia/ml (isolate EABb 01/147-Su). Similarly, the parallelism test showed that the data could be represented by six parallel log concentration-probit lines ($\chi^2 = 7.5$, with 5 *df*) with a common slope (0.36 ± 0.025, mean ± SE). The relative potencies and the 95% fiducial limits of the treatment with isolates EABb 01/110-Su, EABb 93/14-Tp, EABb01/39-Su, EABb 90/2-Dm, and EABb 01/147-Su with respect to the treatment with reference isolate ATCC 74040 were 277 (75–900), 128 (37–530), 23 (8–82), 11 (4–38), and 0.8 (0.2–2.0), indicating again that all isolates, except EABb 01/147, were significantly more virulent to *T. vaporariorum* than reference ATCC 74040 isolate.

Table 2
Regression analysis of probit mortality and log-concentration data of bioassay with different *B. bassiana* isolates against fourth instar nymphs of *B. tabaci* and *T. vaporariorum*

Isolate[a]	*Bemisia tabaci*					*Trialeurodes vaporariorum*				
	No. of insects	Slope (SE)	LC_{50}[b]	95% FL	χ^2 ($df = 2$)	No. of insects	Slope (SE)	LC_{50}[b]	95% FL	χ^2 ($df = 2$)
EABb 01/110-Su	860	0.44 (0.059)	6.53×10^5	2.78×10^5–1.28×10^6	2.004	698	0.51 (0.050)	1.61×10^5	6.07×10^4–3.24×10^5	2.718
EABb 93/14-Tp	918	0.32 (0.056)	1.92×10^6	7.24×10^5–4.46×10^6	3.121	713	0.33 (0.043)	1.08×10^5	1.85×10^4–3.02×10^5	1.188
EABb 90/4-Cb	936	0.39 (0.055)	2.17×10^6	1.02×10^6–4.34×10^6	0.435	—	—	—	—	—
EABb 01/12-Su	916	0.35 (0.054)	2.47×10^6	1.04×10^5–5.47×10^6	2.061	—	—	—	—	—
EABb 91/6-Ci	996	0.30 (0.055)	6.21×10^6	2.57×10^6–1.75×10^7	2.736	—	—	—	—	—
EABb 91/7-Dm	740	0.42 (0.060)	8.64×10^6	4.14×10^6–2.04×10^7	1.820	—	—	—	—	—
EABb 90/2-Dm	750	0.29 (0.059)	2.37×10^7	8.33×10^6–1.21×10^8	1.526	721	0.36 (0.061)	2.32×10^6	9.65×10^5–4.87×10^6	1.572
EABb 01/147-Su	968	0.36 (0.054)	9.34×10^7	3.62×10^7–4.14×10^8	0.685	734	0.32 (0.029)	3.06×10^7	1.17×10^7–1.29×10^8	0.244
ATCC 74040	862	0.21 (0.057)	3.50×10^8	5.64×10^7–4.93×10^{10}	0.079	705	0.31 (0.033)	1.99×10^7	7.98×10^6–7.68×10^7	0.119
EABb 01/39-Su	—	—	—	—	—	743	0.32 (0.065)	6.12×10^5	1.78×10^5–1.45×10^6	0.328

[a] Mortality in the controls ranged between 0 and 3% in virulence assays with both whitefly species.
[b] LC_{50} values and their 95% fiducial limits are expressed in spores per milliliter.

Parallelism test performed with log concentration–mortality data from *B. bassiana* isolates EABb 01/110-Su, EABb 93/14-Tp, EABb 90/2-Dm, EABb 01/147-Su, and ATCC 74040 that were assayed against both whitefly species indicated that, for each isolate, the data from *B. tabaci* and *T. vaporariorum* could be represented by two parallel log concentration-probit lines ($\chi^2 = 0.58$, $\chi^2 = 0.03$, $\chi^2 = 0.76$, $\chi^2 = 0.30$, and $\chi^2 = 1.2$, respectively, with 1 *df*) with a common slope (0.47 ± 0.1, 0.33 ± 0.04, 0.37 ± 0.1, 0.38 ± 0.1, and 0.40 ± 0.1, respectively, mean ± SE). The relative potencies and the 95% fiducial limits of the treatment of *B. tabaci* with isolates EABb 01/110-Su, EABb 93/14-Tp, EABb 90/2-Dm, EABb 01/147-Su, and ATCC 74040 with respect to the same isolates assayed against *T. vaporariorum* were 5 (2–82), 13 (4–63), 10 (1–40), 4 (1–100), and 4 (1–95), respectively, showing that *T. vaporariorum* were significantly more susceptible to all *B. bassiana* isolates than *B. tabaci*.

The second measure of virulence was the AST of treated nymphs that was calculated for the two higher concentrations 1×10^6 and 1×10^7 conidia/ml (Fig. 2). In general, the order of virulence of *B. bassiana* isolates on the basis of AST of treated nymphs was similar for *B. tabaci* and *T. vaporariorum*, whereas AST values were shorter for the latter than for the former species. In *B. tabaci*, only nymphs treated with isolates EABb 01/110-Su and EABb 93/14-Tp exhibited AST values below 7 days at the two conidial concentrations, with AST of 6.8 and 6.2 days and at 1×10^6 and 1×10^7 conidia/ml, respectively. At this higher concentration, isolates EABb 90/4-Cb, EABb 90/2-Dm, and EABb 01/12-Su reduced the AST of nymphs below 7 days, with mean values ranging between 6.7 and 6.9 days (Fig. 2A). Isolates EABb 01/110-Su, EABb 93/14-Tp, and EABb 90/2-Dm were the most virulent to *T. vaporariorum* with AST's at the higher concentration of 5.9, 6.5, and 6.6 days, respectively (Fig. 2B). All isolates, except EABb 01/39-Su and EAB 01/147-Su, showed significantly shorter AST than reference ATCC 74040 isolate in experiments with *B. tabaci* inoculated with 1×10^7 conidia/ml, whereas in those with *T. vaporariorum*, all isolates except EABb 01/147-Su were more virulent than reference isolate ATCC 74040. When the two AST values from the two concentrations were averaged and correlated with the log-LC_{50} for each isolate, it was obtained a correlation coefficient of 0.74 ($P = 0.02$) and 0.95 ($P = 0.03$) for *B. tabaci* and *T. vaporariorum*, respectively, indicating a direct relationship between LC_{50} and AST for both whitefly species.

3.2. Effect of temperature on in vitro radial colony growth of fungi

We selected a set of the eight most virulent isolates against *B. tabaci* and *T. vaporariorum* to study their thermal biology. Temperature had significant effects on in vitro radial colony growth of all fungal isolates (Table 3). The radial measurements from the 2nd to the 10th day fitted a linear model and coefficients of determination of regression lines for all isolates and all temperatures varied from

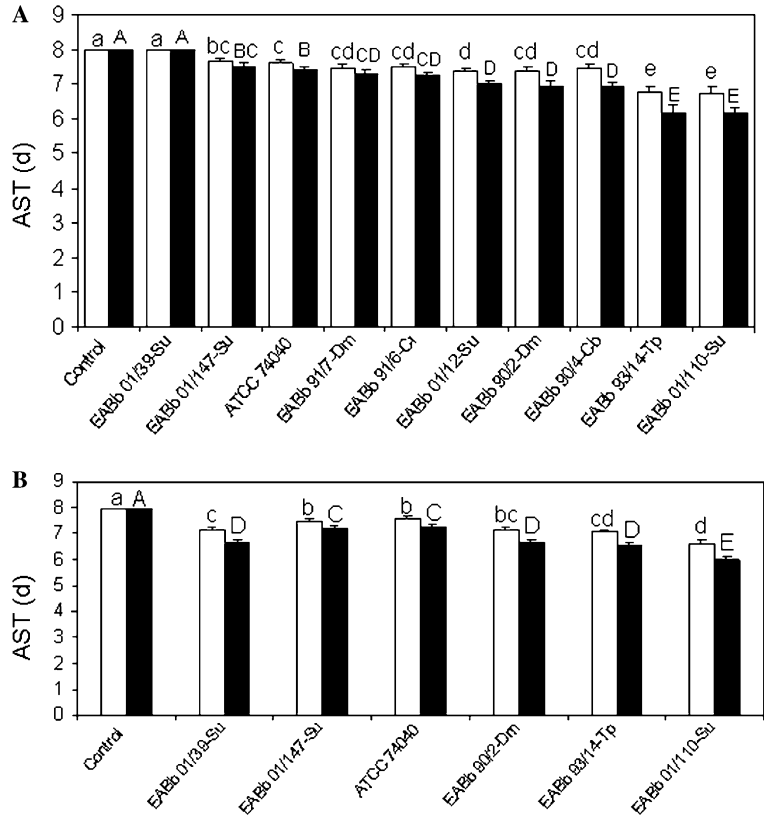

Fig. 2. AST (mean ± SE) of fourth instar nymphs of *B. tabaci* (A) and *T. vaporariorum* (B) treated with 1×10^6 conidia/ml (white bars and lowercase) and 1×10^7 conidia/ml (black bars and capital) of *B. bassiana*. For each concentration, bars not followed by the same letter are significantly different (log rank test $\alpha = 0.05$). AST limited to 8 days.

Table 3
Estimated parameters (±SE) and coefficients of determination r^2 of the generalized β function modified according to Bassanezi et al. (1998)[a] fitted to data of the vegetative growth of different *B. bassiana* isolates

Isolate	TY_{opt}	T_{opt}	T_{max}	TB_3	r^2
EABb 90/2-Dm	4.2 ± 0.5 f	23.9 ± 1.2 ab	34.9 ± 3.5 b	3.1 ± 1.8 c	0.96
EABb 90/4-Cb	3.0 ± 0.3 e	24.6 ± 1.6 bc	31.8 ± 4.8 a	1.0 ± 1.6 b	0.96
EABb 91/6-Ci	1.7 ± 0.2 a	23.9 ± 2.1 ab	35.3 ± 1.0 b	0.8 ± 0.6 b	0.90
EABb 91/7-Dm	1.7 ± 0.5 a	23.1 ± 6.3 a	32.6 ± 0.1 a	0.5 ± 0.4 ab	0.92
EABb 93/14-Tp	2.1 ± 0.1 c	25.1 ± 1.1 c	36.6 ± 1.4 d	0.9 ± 0.4 b	0.97
EABb 01/12-Su	2.3 ± 0.1 d	27.1 ± 0.9 f	35.3 ± 0.3 b	0.5 ± 0.2 a	0.99
EABb 01/39-Su	1.6 ± 0.2 a	25.9 ± 2.1 e	35.9 ± 1.9 c	0.8 ± 0.7 b	0.93
EABb 01/110-Su	2.0 ± 0.1 b	25.7 ± 0.6 d	35.6 ± 0.4 b	0.9 ± 0.2 b	0.99

[a] The generalized β function is given by: $P = TY_{opt}[(T - T_{min})/(T_{opt} - T_{min})] \exp [TB_3 (T_{opt} - T_{min})/(T_{max} - T)] [(T_{max} - T)/(T_{max} - T_{opt})] \exp TB_3$, where $Y(T)$ is the fungal growth in mm per day (dependent variable) and T is the incubation temperature (independent variable). T_{min}, T_{max}, and T_{opt} are respectively the lowest, the highest, and the optimal temperature for fungal growth. TY_{opt} is the fungal growth at the optimal temperature T_{opt} and TB_3 is the shape parameter. Means within columns with the same letter are not significantly different ($P < 0.05$) according to the least significant difference (LSD) test.

0.953 to 0.999. All fungal isolates grew at 10, 15, 20, 25, 30, and 35 °C except isolate EABb 91/7-Dm, which did not grow at 35 °C. Colony extension rates varied from 0.25 to 0.8 mm/day at 10 °C, from 0.9 to 1.7 mm/day at 15 °C, from 1.1 to 2.8 mm/day at 20 °C, from 1.8 to 4.1 mm/day at 25 °C, from 1.1 to 2.7 mm/day at 30 °C, and from 0.2 to 0.9 mm/day at 35 °C. Linear regression slopes indicating the growth rates in mm/day at each temperature were used as the main parameter to evaluate the influence of temperature on fungal growth. The effect of temperature was given by optimum curves, which were on the overall well described by the generalized β function used in our work (Fig. 3 and Table 3). The shape parameter ranged between 0.51 and 0.98 for all isolates except isolate EABb 90/2-Dm, which showed a significantly higher value of 3.18.

MANOVA showed significant differences among isolates in their β model (λ de Wilks = 0.002; $P < 0.001$). Optimum temperatures were significantly different among *B. bassiana* isolates. They varied between 23.1 ± 6.3 °C (EABb 91/7-Dm) and 27.2 ± 0.9 °C (EABb 01/12-Su).

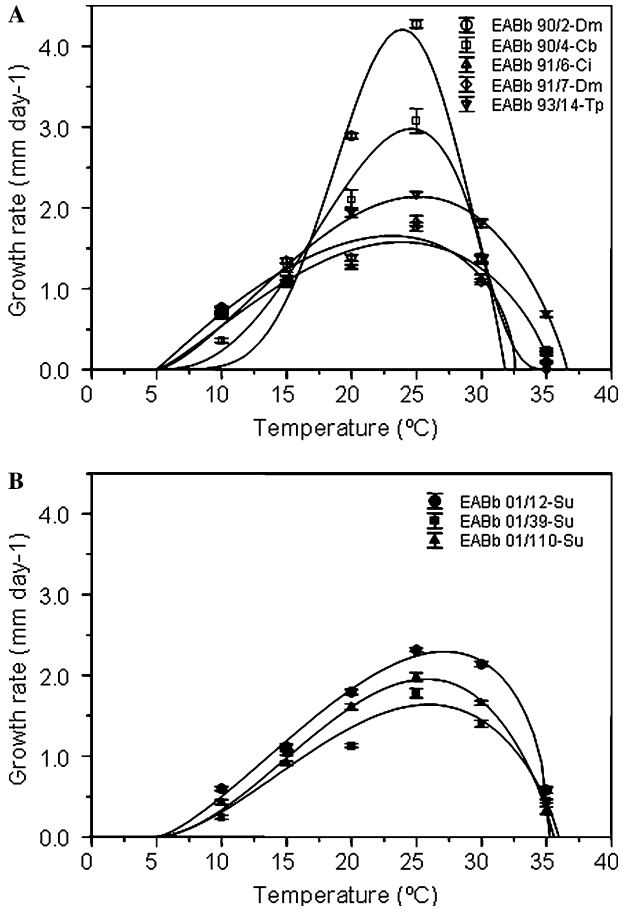

Fig. 3. Model predictions for the effect of temperature on the growth rate of colonies of *B. bassiana* strains isolated from insect hosts (A) and from the soil (B). Lines represent fitted curves obtained using the generalized β function modified according to Bassanezi et al. (1998).

Only four isolates exhibited temperature optima above 25 °C and these were three originating from the soil (EABb 01/12-Su, EABb 01/39-Su, and EABb 01/110-Su) and one from a lepidopteran host (EABb 93/14-Tp). There were significant differences among isolates in maximum temperatures for fungal growth, which varied from 31.8 ± 4.8 °C (EABb 90/4-Cb) to 36.6 ± 1.4 °C (EABb 91/6-Ci). Only two isolates exhibited temperature maxima below 35 °C (EABb 90/4-Cb and EABb 91/7-Dm). Comparisons of relative growth rates at the optimum temperature indicated that there were also significant differences among isolates (Table 3). The growth rates at the optimum temperature varied from 1.6 ± 0.2 mm/day (EABb 01/39-Su) to 4.2 ± 0.5 mm/day (EABb 90/2-Dm). However, growth rate above 3 mm/day was observed only for isolate EABb 90/2-Dm, three isolates exhibited values above 2 mm/day (EABb 93/14-Tp, EABb 01/12-Su, and EABb 90/4-Cb) and the rest of isolates below 2 mm/day.

3.3. Production of insecticidal macromolecules by fungal isolates in Adamek's medium

For toxicological studies, we selected the same isolates as used in the temperature study. In addition, we included three additional isolates (EABb 0029-Su, EABb 01/145-Su, and EABb 01/15-Su) that were classified as least virulent for both species. Filtrates from all isolates, precipitated with ammonium sulfate and desalted on G-25, had a significant toxic effect when injected into *G. mellonella* larvae ($F_{11,84} = 520$, $P < 0.001$ for 8 μg of total protein/insect). Mortality of *G. mellonella* larvae recorded 7 days post-injection varied from 42 to 99%, as compared with mortality of 3.8% for controls (Fig. 4A). G-25 fractions from all isolates caused mortality rates higher than 90% except EABb 00/29-Su (42.5%) and EABb 91/6-Ci (51.6%). Microscopic observation of tissues from cadavers revealed the following signs: (1) melanized dark spots on tracheae and air sacs (G-25 fractions from isolates EABb 91/6-Ci, EABb 91/7-Dm, and EABb 93/14-Tp); (2) melanization of the cuticle with pigmented individualized spots (fraction from isolate EABb 90/2-Dm); (3) signs 1 and 2 together (fraction from isolate EABb 01/110-Su). Fractions from isolates EABb 01/39-Su and EABb 01/12-Su caused a heavy larval paralysis after injection leading to dead.

The AST of larvae injected with G-25 fractions from all isolates varied from 1.3 to 6.3 days compared with 7 days for controls (Fig. 4B). Different groups could be distinguished among G-25 fractions according to their toxicity: a first group of weakly toxic fractions (AST of injected larvae above 6 days), a second intermediate group (AST of injected larvae between 3 and 5 days) and a third highly toxic group which reduced AST of injected larvae below 3 days. Among G-25 fractions from the intermediate group, no significant differences were observed in AST between that from isolate EABb 01/110-Su (AST 3.7 ± 0.4 days) and those from isolates EABb 90/2-Dm (AST 4.6 ± 0.4 days) (log rank = 2.34; $P = 0.1263$) and EABb 93/14-Tp (AST 3.6 ± 0.4 days) (log rank = 0.13; $P = 0.7164$). Among G-25 fractions within the highly toxic group, that from isolate EABb 01/12-Su was the most toxic one (AST 1.4 ± 0.1 days) compared with those from isolates EABb 01/39-Su (1.9 ± 0.2 days) (log rank = 5.97; $P = 0.0146$) and EABb 01/15-Su (AST 2.7 ± 0.2 days) (log rank = 20.96; $P < 0.001$). To pursue the analysis, G-25 fractions from isolates EABb 01/12-Su, EABb 01/110-Su, EABb 93/14-Tp, EABb 01/15-Su, and EABb 01/39-Su were submitted to proteinase K treatment. When control solutions of proteinase K at 1 mg/ml were injected into larvae, the treated insects did not exhibit any sign of cuticular melanization or any other toxic effect, and the rate of mortality was <5%. Treatment of G-25 fractions from all isolates with proteinase K caused a decrease of their lethal effects. The protease treatment had a significant effect on larval mortality ($F_{11,84} = 1066$, $P < 0.001$). Mortality rates caused by all fractions were significantly higher (Tukey test, $P < 0.001$) before (77.5–97.0%) than after enzymatic treatment (1.9–25.0%), with percentage reduction ranging between 74.1 and 98.0%. Moreover, the protease treatment significantly (log rank test $\alpha = 0.05$) lengthened the AST of injected larvae by 33.0–64.7%, with mean values varying from 2.4 to 4.1 days before enzymatic

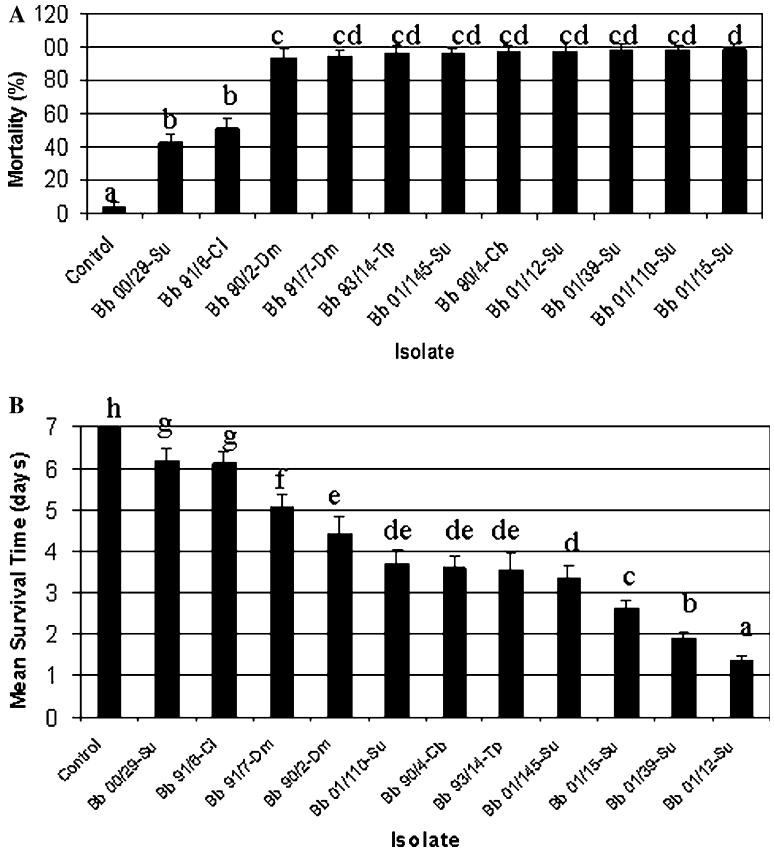

Fig. 4. (A) Percentage mortality (mean ± SE) of fourth instar *G. mellonella* larvae injected with 8 μg of total protein per insect of the Sephadex G-25 desalted fraction of 11 *B. bassiana* strains. Bars with the same letter are not significantly different according to the Tukey (HSD) test ($P < 0.05$). (B) AST of fourth instar *G. mellonella* larvae injected with 8 μg of total protein per insect of the Sephadex G-25 desalted fraction of 11 *B. bassiana* strains. Bars with the same letter are not significantly (log rank test $\alpha = 0.05$).

treatment and from 5.4 to 6.8 days after it. In addition, proteinase K treatments inhibited the development of any sign of melanization and paralysis.

4. Discussion

The first criterion of our biorational approach for selecting isolates of *B. bassiana* for whitefly control was high virulence against the target organism. In our work, all isolates of *B. bassiana* that were assayed against fourth instar nymphs of *B. tabaci* were pathogenic. However, they showed large differences in virulence, only the seven isolates within the third highly virulent group (none of which was the reference ATCC 74040 isolate) caused mortality rates >50%. Among them, there was only one isolate (EABb 01/110-Su) causing mortality above 70% (77.7%), and two isolates (EABb 93/14-Tp and EABb 90/4-Cb) causing mortality rates above 60%. These isolates were also the most virulent against *T. vaporariorum*, although mortality against *B. tabaci* was less than against *T. vaporariorum*. This result agrees with that obtained by Meekes et al. (2002) with *Aschersonia* sp. isolates, who found total mortality to be higher for *T. vaporariorum* than for *Bemisia argentifolii* Bellows and Perring due to differences in mortality in the control treatments or mortality due to unknown causes. We did not observed any differences in the control mortality between both species and the waxy filaments present in the greenhouse whitefly nymphs but not in *B. tabaci* could lead to greater conidial retention and could explain the greater infection found in the former species.

In our work, mortality levels of *B. tabaci* and *T. vaporariorum* were similar to those caused by *Aschersonia* spp. (Meekes et al., 2002), but less than mortality levels caused by *B. bassiana* and other fungi like *Metarhizium anisopliae* (Metsch.) Sorokin, *P. fumosoroseus* and *Verticillium lecanii* (Vuill.) found by other authors, viz., between 70 and 95% mortality (Gindin et al., 2000; Malsam et al., 1998; Vidal et al., 1997; Wraight et al., 1998). This comparison, however, should be interpreted with care, since fungal isolates used in the latter studies were passed through whitefly before experimentation while our isolates had no passage through a host. For most entomopathogenic fungi passage through the host enhances virulence and repeated subculturing on artificial medium can decrease it (Brownbridge et al., 2001; Quesada-Moraga and Vey, 2003). The pronounced red pigmentation displayed by nymphs of both species infected with *B. bassiana* has been also described by Wraight et al. (1998) in nymphs of *B. argentifolii* infected with the same fungal species and may be due to the production of the red pigment oosporein (Vey et al., 2001).

We selected the most virulent isolates against both whitefly species together with representative isolates showing lower efficacy for a second series of virulence bioassays. The low regression coefficients obtained in our work could be due to a certain degree of heterogeneity of the population in susceptibility to fungal infection that may cause a slower rise in mortality associated with a given increase in concentration. In general, the regression coefficients provided by other authors working with whiteflies are not very high. By using probit analysis, Wraight et al. (1998) obtained regression coefficients ranging from 0.5 to 2.0 for *Paecilomyces* spp. and *B. bassiana* isolates inoculated to third instar *Bemisia argentifolii*, whereas Vidal et al. (1997) obtained slopes varying from 0.62 to 1.13 for second instar *B. argentifolii* inoculated with *P. fumosoroseus*. Recently, Wang et al. (2004) have provided slopes ranging between 0.70 and 0.85 using CLL time–concentration–mortality model for six strains of *V. lecanii* against third instar sweetpotato whitefly, *B. tabaci*. Difference among regression slopes between these works and our work could be explained partially by the difference of susceptibility in nymphal stages of the whiteflies as demonstrated by Gindin et al. (2000), who observed the higher virulence of *V. lecanii* to occur against newly emerged nymphs and to be progressively reduced in the other instars, with adults being less susceptible to fungal infection. Apart from the issue of the low slopes and for being more resistant to fungal infection than lower instars, using fourth instar whiteflies for selection of fungal strains seems to guarantee the efficacy of the fungus against all instars.

Isolates EABb 01/110-Su and EABb 93/14-Tp were the most virulent for *B. tabaci* (LC_{50} 6.5×10^5 and 1.9×10^6 conidia/ml, respectively) and *T. vaporariorum* (LC_{50} 1.6×10^5 and 1.1×10^5 conidia/ml, respectively). All isolates, except EABb 01/147-Su, were more virulent than reference isolate ATCC 74040, with potency ratios varying from 4 to 119 for *B. tabaci* and from 11 to 278 for *T. vaporariorum*. The virulence of isolates assayed against both whiteflies was 4–13 times higher to *T. vaporariorum* than to *B. tabaci*. Most importantly, our parallelism tests suggested that the pattern of response to increasing concentrations was similar among isolates assayed against each whitefly species and among those assayed against both species.

There are some reports on virulence of *B. bassiana* against *Bemisia* spp. and *T. vaporariorum* that may be compared to those presented here because they used similar assay protocols. Eyal et al. (1994) inoculated *B. argentifolii* nymphs by dipping for 10 s in *B. bassiana* and *P. fumosoroseus* conidial suspensions and reported 52–98% mortality following treatments with concentrations of approximately $1–4\times10^6$ conidia/ml, although LC_{50}s were not calculated. We have found LC_{50}s of the most virulent isolates to both whitefly species to range between 1.8×10^5 and 8.6×10^6 conidia/ml, which approximates results for the strains used by Eyal et al. (1994). Wang et al. (2004) obtained LC_{50} values of 2.5×10^5 and 6.0×10^5 conidia/ml for two *V. lecanii* isolates screened for virulence against *B. tabaci*, while Hernández and Garza (1994) reported a LC_{50} of 4.3×10^8 conidia/ml for *P. fumosoroseus* isolate PF2 against *B. tabaci*, which is larger than that of our best values but it is in the range for our weaker isolates. On the other hand, from our estimations based on the use of the colony forming unit (CFU) method to obtain approximate number of conidia per mm^2, LC_{50} values of our most virulent isolates to *B. tabaci* and *T. vaporariorum* varied from 100 to 1000 conidia/mm^2, which approximate the values provided by Wraight et al. (1998) (350–4000 conidia/mm^2) and Vidal et al. (1997) (619–1269 conidia/mm^2) using third and second instar nymphs of *B. argentifolii*, respectively.

The virulence of a given fungal isolate is not only indicated by LC_{50} but also by the time in days required to achieve 50% mortality (LT_{50}) or by the AST. Surprisingly, LT_{50} or AST values have not been reported by any of the previous authors except Gindin et al. (2000), who obtained LT_{50} values of 3–4, 4–7, and more than 7 days for the high, moderate, and weakly virulent *V. lecanii* isolates, respectively, at a concentration of 1×10^7 conidia/ml. In our work, we have found a high correlation between LC_{50} and AST values for both whitefly species. Particularly, isolates EABb 01/110-Su and EABb 93/14-Tp were the only ones that reduced the AST of nymphs of both whitefly species below 7 days at the two conidia concentrations with values of 6.2 days for *B. tabaci* and 6.0–6.5 days for *T. vaporariorum* at 1×10^7 conidia/ml. In comparison, the AST of nymphs inoculated with isolates EABb 01/39-Su, EABb 01/147-Su, EABb 91/7-Dm, EABb 91/6-Ci, and ATCC 74040 never was below 7.3–7.5 days. Hypothetically, under field conditions there would be more opportunity for population increases in whiteflies treated with any of the latter isolates (i.e., isolate ATCC 74040) compared with EABb 01/110-Su and EABb 93/14-Tp. Moreover, whiteflies inoculated with reference ATCC 74040 isolate would potentially be available over a longer period to produce direct and indirect damages and to reach the adult stage before succumbing to fungal infection. What is certain is that isolates EABb 01/110-Su and EABb 93/14-Tp killed the nymphs faster than the commercial reference ATCC 70874 isolate, which is currently being used as a reliable mycoinsecticide for integrated whitefly control in some Mediterranean countries as Spain (de Liñan, 2005).

The temperature regimes of greenhouses could have a significant impact on mycoinsecticide activity, which makes necessary a thorough understanding of the effects of temperature on fungal development. For this reason, we measured the response of the most virulent isolates to the medium and high temperatures that are likely to be encountered in Mediterranean greenhouses in spring and summer. The colony extension technique employed here is used widely to estimate the optimum temperatures for fungal growth and is a valuable tool for screening entomopathogenic fungi that have already demonstrated high virulence against the target pest (Davidson et al., 2003; Fargues et al., 1992; Mietkiewski et al., 1994; Ouedraogo

et al., 1997; Vidal et al., 1997; Yeo et al., 2003). The response of fungi to temperature in a characteristic bell-shaped curve was skewed to the lower temperatures. However, non-linear approaches to analyzing entomopathogenic fungus–temperature interactions are not yet used routinely.

To our knowledge, we have used, for the first time, the generalized β function modified according to Bassanezi et al. (1998) to study temperature–growth interactions of entomopathogenic fungi. Previous works in invertebrate mycopathology have used the Sharpe and DeMichele (1977) model of poikilotherm development (Patel et al., 1991; Smitley et al., 1986), the Schoolfield et al. (1981) re-formulation of the Sharpe and DeMichele model (Davidson et al., 2003), or other non-linear regression procedures (Ouedraogo et al., 1997; Thomas and Jenkins, 1997). The most important advantage of using the generalized β function modified according to Bassanezi et al. (1998) is that all parameters have biological meaning while in some other models there are some parameters that has not such characteristic. The temperature growth responses varied considerably between our *B. bassiana* isolates, with some having wide ranges of temperatures around the optimal temperature (higher shape parameter TB_3 values) while others were much more restricted. Differences in absolute and relative growth rates between isolates could be distinguished at all temperatures as observed by Fargues et al. (1997) for 65 *B. bassiana* isolates from different geoclimatic and host origins. The optimal temperature of our isolates ranged between 23.1 and 27.1 °C in accordance that (25–28 °C) observed by Fargues et al. (1997). Moreover, the upper thermal limit of our isolates (31.8–36.6 °C) also approximates the one obtained by Fargues et al. (1997) for their *B. bassiana* isolates (35–37 °C).

In developing a mycoinsecticide for control of whiteflies in Mediterranean greenhouses, it is especially important to evaluate the action of candidate fungi at high temperatures that may be limiting in the field. This is particularly true in southern Spain, where greenhouse ambient maximum temperatures may be over 35 °C for some hours per day in summer. However, leaf surface temperatures are undoubtedly lower than ambient (Willmer, 1986) and that during spring and fall seasons the temperatures are generally more favorable for these fungi. If both the optimal temperature for growth (25–27 °C) and the upper thermal limit (35.3–36.6 °C) are considered, three of our more virulent isolates, EABb 01/110-Su, EABb 93/14-Tp, and EABb 01/12-Su, showed thermal requirements slightly better matched to those conditions. Yet, the thermal biology of insects in greenhouses is extremely complex, and it still remains to be determined whether isolates possessing slightly higher thermal thresholds in vitro will translate into increased efficacy in field conditions. For that, it is necessary to know the epizootic potential of these isolates under greenhouse conditions with particular emphasis on the effects of the high temperatures on the germination and infection processes.

If mycoinsecticides are to compete with chemical pesticides, overall ecological fitness is not the only requirement for an effective mycoinsecticide, but also high speed of kill. Our results indicated that with the exception of isolates EABb 00/29-Su, EABb 91/6-Ci, and EABb 91/7-Dm, there were no significant differences between mortality and ASTs caused by G-25 fractions from weakly, moderately, and highly virulent isolates, demonstrating that in vitro production of macromolecular compounds toxic to *G. mellonella* larvae was not a requisite for virulence, as reported by Kershaw et al. (1999) for destruxins and by Maimala et al. (2002) for Hirsutellin A. However, our objective was to reveal the potential of candidate isolates to be further improved through biotechnology to kill the pest more quickly. To this end, three main requirements are highly desirable for strain selection. The first is the secretion in vitro of highly toxic compounds. The second is for these compounds to be proteins. The third is for these insecticidal proteins to be virulence factors. Our work showed that isolates EABb 01/110-Su, EABb 93/14-Tp, and EABb 01/12-Su met at least the two first requirements. ASTs of *G. mellonella* larvae injected with G-25 fractions of these isolates were 3.7, 3.6, and 1.4 days, respectively, compared with 5–6 days for non-toxic fraction. The insecticidal effect of the Sephadex G-25 fractions from these isolates was significantly reduced by proteinase K treatment, suggesting that they were proteinaceous. However, more research is needed to purify the active proteins and to address the issue of whether or not in vitro insecticidal compounds produced by isolates EABb 01/110-Su, EABb 93/14-Tp, and EABb 01/12-Su, are virulence factors.

The *Galleria* assay is therefore useful as a primary screen to evaluate whether or not the selected isolates could be further improved through biotechnology. The first step to increase fungal virulence and improve commercial efficacy is to optimize production of the inoculum (Wraight et al., 2001). Moreover, it has been demonstrated that toxicogenic activity of fungal proteic macromolecules is dependent on the mycological media the inoculum is produced on (Quesada-Moraga and Vey, 2003). But it is also a very promising approach to add additional copies of the genes encoding for these insecticidal proteins, and hopefully by overproducing them, as already performed by St. Leger et al. (1996).

Beauveria bassiana isolates, EABb 01/110-Su, EABb 93/14-Tp, and EABb 01/12-Su, were the most virulent to *B. tabaci* and *T. vaporariorum* and had thermal requirements better matched to the temperatures in Mediterranean greenhouses than the rest of the tested isolates. These characteristics make them good candidates for developing a mycoinsecticide for the control of *B. tabaci* and *T. vaporariorum*. In addition, we have shown that these isolates secreted in vitro highly toxic proteic compounds. The purification of these compounds will allow us to study whether they are also produced in vivo as virulence determinants and their potential to be further improved through biotechnology to kill the pest more quickly. An additional aim

from an applied point of view is the evaluation of the potential of these toxic fungal compounds as new bioinsecticides for whitefly control as previously revealed by Davidson et al. (1996) for destruxins produced by *M. anisopliae*.

In summary, a biorational approach is based on selecting isolates on a range of characteristics. These include high virulence to target host, the ability to grow quickly in vitro at temperatures under which the mycoinsecticide will be used, and the potential for biotechnologically improving their virulence and speed of kill. In summary, these characteristics will aid in selecting fungal isolates that will be more reliable as mycoinsecticides for use in whitefly control in Mediterranean greenhouses.

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