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ORIGINAL PAPER

Substrate influence on physiology and virulence of *Beauveria bassiana* acting on larvae and adults of *Tenebrio molitor*

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Abstract Two isolates of *Beauveria bassiana*, wild type (*wt*) and its mutant type (*mt*) were compared in terms of growth patterns on culture plates containing media based on wheat bran, grasshopper exoskeletons, colloidal chitin or Sabouraud-dextrose agar (SDA). Germination for the *mt* isolate was up to 33% faster in all media. Influence of media on virulence was determined against larvae and adults of *Tenebrio molitor*. Mortality higher than 90% was reached for adults after 6 days using conidia from all media. For larvae, a mortality of 80% was reached after 11 days with conidia collected from SDA medium and between 15 and 35% with conidia from other media. In SDA medium, conidial yield was almost ten times higher for the *mt* isolate compared to the *wt* isolate; however, virulence traits were similar against either larvae or adults. These results may influence commercial preparations of entomopathogenic fungi based on conidia.

Keywords *Beauveria bassiana* · Growth patterns · Nutrient effect · Virulence · *Tenebrio molitor*

Introduction

It is well known that synthetic insecticides create deleterious effects in the environment worldwide; as a consequence attention has shifted to biocontrol agents as suitable alternatives (Safavi et al. 2007). The entomopathogenic fungus (EF) *Beauveria bassiana* (Balsamo) Vuillemin has been extensively used for the control of many pests invading crops around the world. This fungus seems to have a high genetic variability in order to adapt to changing environmental conditions and also to attack, successfully, different insect populations (Bidochka et al. 2002). During the infection of insects by EF, a series of systematic interactions occur between the fungus and the host, including, attachment to the cuticle, germination and production of cuticle degrading enzymes (Hajek and St. Leger 1994).

An insect commonly used in studies of EF–host interaction is the Yellow Mealworm Beetle: *Tenebrio molitor* L. which appears all over the world in reservoirs containing, grains, flour, tobacco, and foodstuffs; both larvae and adults cause considerable material damage (Sallam 2008). Possible factors of developmental stage susceptibility are difficult to establish for a broad range of entomopathogenic microorganisms including *B. bassiana*. Despite that, changes in susceptibility could be attributed to anatomic differences in the integumental structures, respiratory tract and also to differences in the immune response (Schmid-Hempel 2005; Vestergaard et al. 1999).

Differences in the virulence of EF can be related to nutritional factors and to environmental growth conditions prevailing through the stage of spore production (Shah et al. 2005). Despite the facts that fungal nutritional requirements might be strain dependent, some researchers have found some general effects of the carbon and nitrogen sources on virulence parameters. For example, Santoro et al. (2007) found that *B. bassiana* conidia grown on insect cadavers showed less virulence than those produced on rice or synthetic media. Rangel et al. (2004) demonstrated that conidia of *M. anisopliae* var. *anisopliae* produced on insect cadavers germinated slower than conidia produced on a rich artificial medium.

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In this work, two isolates of *B. bassiana* were grown on culture media supplemented with different carbon and nitrogen sources; this led to determine the influence of media composition on both growth patterns and conidial virulence against larvae and adults of *T. molitor*. This allowed the identification of the most effective culture medium in terms of production levels and virulence of conidia acting on adult and larval stages of *T. molitor*.

Materials and methods

Cultures and microbial propagation

The wild type (*wt*) isolate of *B. bassiana* belongs to the fungal collection of the Postgraduate College in Texcoco, Mexico. It was isolated from *Hypothenemus hampei* (Ferrari) in Oaxaca, Mexico. The mutant type isolate (*mt*), was previously obtained by Robledo-Monterrubio et al. (2005) as a deoxyglucose resistant isolate. Inocula for all experiments were conidia harvested using a 0.01% solution of Tween 80, from Sabouraud-dextrose agar cultures propagated at 28°C for 10 days. Conidial concentration was determined using a Neubauer hemocytometer.

Preparation of substrates and media

Colloidal chitin was prepared according to Hsu and Lockwood (1975). Cuticles of adult *Sphenarium purpurascens* were processed according to Barranco-Florido et al. (2002). Commercial wheat bran and cuticle particles were milled separately, and then each material was put through a sieve in order to obtain particles with a diameter between 0.52 and 0.8 mm. Cultures on petri plates were prepared using the mineral medium reported by Barranco-Florido et al. (2002) and mixed with 15 gl^{-1} of agar. This was supplemented with different substrates as sole source of carbon and nitrogen as follows, colloidal chitin (CC) 20 gl^{-1}, *S. purpurascens* particles (SPP) 20 gl^{-1}, wheat bran (WB) 10 gl^{-1}. Agar plates based only on Sabouraud dextrose agar (40 gl^{-1} glucose, 10 gl^{-1} peptone) were labeled as SDA. Initial pH values for all media were adjusted to 7. Incubation was kept at 28°C for all media.

Conidial germination

Petri plates were inoculated with an aliquot of 50 µl of a conidial suspension (1×10^6 conidia ml^{-1}) which was spread out over the plate surface with a sterile glass rod. Plates were sealed with Parafilm and incubated at 28°C. Two agar plugs, 1 cm diameter, were taken every 2 h starting at 10 up to 24 h of incubation; these samples were prepared for microscopic observations by mounting on slides and staining with a drop of lactophenol cotton blue on the agar surface in order to ease identification of germinated conidia (Abbas et al. 1995). Percentage of germination was estimated by counting two fields of 100 spores for each plug, then time to reach 50% germination (TG_{50}) was calculated (Varela and Morales 1996). A spore was considered to have germinated if germ tube length was greater than the spore diameter (Ibrahim et al. 2002).

Growth measurements

Petri plates were point inoculated in the center with a sterile wood toothpick previously immersed for 3 s in a conidial suspension containing 5×10^5 conidia ml^{-1}, plates were sealed with Parafilm and incubated at 28°C. Radial growth rate, U_r, was determined by measuring colony diameter every 24 h for 10 days. Three replicas were analyzed for every media and for each isolate. At the end of those measurements, colonies were observed under the microscope with a digital camera linked to an image processor. The software Imagenia 2000 was used to make fifteen measurements and obtain average data for both hyphal length and hyphal diameter, which were used to estimate specific growth rate, μ, according to the growth model for filamentous fungi described by Loera and Viniegra-González (1998).

$$\mu = \frac{U_r \ln(2)}{L_{av} \ln\left(\frac{L_{av}}{D_h}\right)}$$

where U_r, was the maximal rate of extension of leading hyphae (µm day^{-1}); L_{av}, was the average length of leading hyphae in the periphery of studied colonies (µm); D_h, was the mean diameter of hyphal tubules (µm).

Sporulation

Fifty microlitres of a conidial suspension (5×10^6 conidia per plate) were spread on the agar plates with a sterile glass rod, and subsequently plates were sealed with Parafilm and incubated at 28°C. Three agar plugs (diameter: 1 cm) were taken from each plate and examined at day 6, after collecting conidia with a solution of 0.01% Tween 80 (Kamp and Bidochka 2002). Mean conidial yield per square centimeter was calculated, and referred to as conidial density, ρ. (Varela and Morales 1996).

Virulence bioassay

Inocula obtained from the Petri plates from each media were assayed separately against larvae or adult insects of *T. molitor*. Insects were immersed for 3 s in a 10 ml conidial suspension (1×10^8 conidia ml^{-1}). A 0.01%

solution of Tween 80 without conidia was used as a control. Experimental units consisted on Petri plates containing 12 insects, either larvae or adults, and having oat flakes as food. The plates were kept at 28°C, with a 12:12 h photoperiod. Adults were monitored for 6 days and larvae for 11 days. Three replicas were made for each treatment. Mortality was recorded daily and dead insects were transferred to moist chambers to encourage external sporulation in order to confirm that death was due to fungal infection (Shah et al. 2005). The time to reach 50% of mortality, LT_{50}, was estimated from a plot of accumulated mortality versus time. Data were fitted to an exponential decay function, as follows:

$$Y = 100; \quad \text{If } 0 \leq t \leq t_0$$

$$Y = (100 - S)e^{-k(t-t_0)} + S; \quad \text{If } t > t_0$$

where Y, is the percent of survival at time t; k, is the specific death rate (day^{-1}); t_0, is the delay time (d) and, S, is the estimated asymptotic survival level (%). This model corresponds to the solution of a first order differential equation with the indicated time delay, the aforementioned initial condition and the asymptotic value, $Y \rightarrow S$, for $t \rightarrow \infty$

Statistic analysis

Data were analyzed using one-way analysis of variance (ANOVA) and the Tukey least significant difference test at $P < 0.05$. A statistical analysis software (NCSS) was used.

Results

Characterization on petri plates

Table 1 shows a summary of physiological results on petri plates. Radial growth rates, U_r, for the *mt* isolate were between 6 and 18% higher than for the *wt* isolate, on all media except on SDA. Varela and Morales (1996) observed U_r values between 2.1 and 2.7 mm day^{-1}; and Safavi et al. (2007) found values of U_r from 2.9 up to 4.1 mm day^{-1}, in both cases growing *B. bassiana* on SDA medium. The microscopic observation of *B. bassiana* mycelium showed that hyphal diameters in all media were close to 3 μm, with the exception of hyphal diameters of the mold grown in medium CC (1.7 μm). The hyphal length for the *wt* isolate in complex media, such as SPP and WB had an average value around 360 μm; whereas, in CC and SDA this value was 36% shorter. Differences in this parameter among isolates were not statistically significant, except in the medium SPP, where significant differences indicated a major degree of branching in the *mt* isolate respect to the *wt* isolate. The specific growth rate, μ, showed that differences between isolates are magnified when grown on SPP and WB media (45 and 32%, respectively) as compared to differences between isolates grown on SDA and CC (16 and 18%, respectively), nevertheless those differences were not significant. Furthermore, the time to reach 50% germination, TG_{50}, was consistently shorter (between 20 and 36%) for the isolate *mt*, as compared to *wt* isolate in all media. Varela and Morales (1996) reported a TG_{50} from 15 up to 19 h for *B. bassiana* grown on SDA; these values are similar to those obtained for the *wt* isolate in this study.

Conidial yield in SDA medium obtained after 6 days of inoculation for the *mt* isolate was almost ten times higher than the value reached by the *wt* isolate. In the same medium, Kamp and Bidochka (2002) obtained a production of 0.97×10^7 con cm^{-2} after 14 days of culture, which is three times and 28 times lower than values achieved by *wt* and *mt* isolates, respectively. On the other hand, Safavi et al. (2007) obtained a value (3×10^7 con cm^{-2}), very similar to that reached by our *wt* isolate in SDA. However, in a medium with similar composition to CC, isolates used in this study (*wt* and *mt*) produced seven and two times more, respectively, than isolates used by Safavi et al. (2007). It is worth noting the low values for U_r and D_h were observed for both isolates, as well as a low value of ρ, L_{av} and μ for the *mt* isolate grown on CC medium. Such data indicate that both isolates had noticeable phenotypic growth and sporulation differences among themselves.

Insect virulence bioassay

Table 2 shows a summary of results on the virulence bioassays. There were large differences of mortality when the assays were performed with larvae as compared to adults. For conidia harvested from culture media CC, SPP, and WB mortality of larvae was lower than 40% after 11 days; a 50% of mortality was only achieved after 6 days with conidia from SDA medium for both isolates. In the case of the assays with adults, 50% of mortality was achieved in less than 3 days. This preliminary analysis indicated a much higher vulnerability of adult stage as compared to the larval stage. A more refined analysis was done using the model indicated before. The asymptotic survival value, S, was close to zero in all the tested cases for adults (100% final mortality) and was much higher for larvae (asymptotic mortality lower than 86%). Moreover, conidia obtained from CC, SPP and WB media, achieved an average S value, significantly different, which was four times higher than the value obtained with SDA medium ($S = 17.5\%$). The decay curve started, approximately, after 3 days of inoculation, for most of the cases. Furthermore, the decay rate constant, k, showed to be statistically similar

Table 1 Comparison of physiological parameters on Petri plates between isolates, for the wild type (*wt*) and mutant type (*mt*) isolate

Parameter	Media			
	SDA	CC	SPP	WB
U_r (mm day^{-1})				
wt	2.2 ± 0.1^{a}	1.4 ± 0.01^{b}	2.2 ± 0.04^{ac}	1.8 ± 0.2^{d}
mt	2.2 ± 0.01^{a}	1.5 ± 0.0^{b}	2.5 ± 0.06^{c}	2.2 ± 0.05^{a}
L_{av} (μm)				
wt	232.5 ± 62^{ab}	233 ± 55^{ab}	392.5 ± 122^{e}	330.7 ± 74^{d}
mt	254 ± 55^{abc}	200.5 ± 38^{a}	295.7 ± 65.6 ^{cd}	281 ± 66^{bcd}
μ (h^{-1})				
wt	0.06 ± 0.01^{b}	0.04 ± 0.006^{ab}	0.03 ± 0.003^{a}	0.034 ± 0.006^{a}
mt	0.055 ± 0.006^{ab}	0.045 ± 0.00^{ab}	0.055 ± 0.012^{ab}	0.05 ± 0.006^{ab}
TG_{50} (h)				
wt	14.4 ± 0.1^{c}	17.8 ± 0.05^{d}	17.8 ± 0.1^{d}	15.2 ± 0.2^{c}
mt	9.9 ± 0.7^{a}	13 ± 0.5^{b}	14.5 ± 0.3^{c}	9.7 ± 0.1^{a}
ρ (Conidia cm^{-2}) (10^7)				
wt	3.1 ± 1.8^{a}	7.2 ± 1.3^{a}	25 ± 5.6^{b}	21 ± 4.4^{b}
mt	28 ± 3.8^{b}	2 ± 0.36^{a}	6.6 ± 4.4^{a}	20 ± 2.7^{b}

U_r, radial growth rate, mm day^{-1}; L_{av}, average hyphal length, μm; μ, specific growth rate, day^{-1}; TG_{50}, time to reach 50% of germination, h; ρ, conidial density, conidia cm^{-2}, was evaluated at 6 days of culture. *SDA* Sabouraud-Dextrose agar, *CC* Colloidal chitin, *SPP Sphenarium purpurascens* particles, *WB* wheat bran. Means with the same letter were not statistically different

for assays on larvae with conidia obtained in all culture media ($k = 0.6$ day^{-1}), and statistically different to the average value with adults. Among the assays with adults, average k value was almost three times higher for the conidia grown on SDA medium ($k = 4.1$ day^{-1}) than those obtained in the other culture media. This seems to indicate that adults are killed at a faster rate than larvae and also that, conidia from SDA medium killed faster the adult organisms. Apparently, the culture medium SDA is a better choice to induce more virulence of *B. bassiana* against *T. molitor* than the other more complex media (CC, SPP or WB). It is worth noting that fungal infection of dead insects was confirmed in moist chambers, final mortality for control experiments was lower to 1% level.

Discussion

This work has two main results, namely, adults of *T. molitor* were more susceptible than larvae to the attack by *B. bassiana*. Also, Sabouraud medium (SDA) yielded more virulent spores than complex media made of chitin (CC), Grasshoppers exoskeletons (SPP) or wheat bran (WB). As far as we know, this is the first report of a major susceptibility of adult insects as compared to larvae of *T. molitor* when infected by conidia of *B. bassiana*, although these results differ to previous virulence assays achieved on insects of the Tenebrioidae family. Rohde et al. (2006) and Alexandre et al. (2006) indicated that isolates of *B. bassiana* were more efficient against larvae than against insect adults of the lesser mealworm *Alphitobius diaperinus*. Rohde et al. (2006) reported variable mortalities after 10 days of bioassay: 7–100% for larvae and 0–86% for adults; whereas Alexandre et al. (2006) reported mortalities between 57 and 89% against adults after 10 days, lower than those achieved in our study, where 100% mortality using adults of *T. molitor* was reached between 4 and 6 days.

Differences of infection related to developmental stage could be associated to the nature of the coleopteran adult cuticle surface and the presence of some external substances, such as mucilage, which may enhance the attachment of conidia (Hajek and Leger 1994). Additionally, the presence of membranes under the elytra, and the abundance of spiracles, pore canals, and intersegmental spaces could facilitate mycelia penetration in adults as compared to larvae. Another important factor may be related to ecdysis, since Vestergaard et al. (1999) described that conidia were undetected in new cuticles after larvae molting. Other reason for higher susceptibility to infection in adults could be a decrease in the immune response related to age, linked in turn to a major energy investment on reproduction than on defense. This has been related also to phenoloxidase activity (PO) and encapsulation rate (Schmid-Hempel 2005).

The influence of the culture medium on the virulence has been reported by some authors (Ibrahim et al. 2002; Rangel et al. 2004; Shah et al. 2005). In the case of

Table 2 Virulence bioassay for the wild type (*wt*) and mutant type (*mt*) isolate

Parameter	Larvae				Adults			
	SDA	CC	SPP	WB	SDA	CC	SPP	WB
LT_{50} (day)								
wt	6.1 ± 1.3^{a}	NR	NR	NR	2.4 ± 0.2^{c}	2.6 ± 0.0^{c}	2.5 ± 0.1^{c}	2.3 ± 0.05^{c}
mt	5.7 ± 1.5^{a}	NR	NR	NR	2.6 ± 0.2^{c}	2.5 ± 0^{c}	3.6 ± 0.02^{cd}	4.1 ± 1^{d}
k (day^{-1})								
wt	0.9 ± 0.3^{a}	0.6 ± 0.06^{a}	0.6 ± 0.16^{a}	0.5 ± 0.06^{a}	3.7 ± 0.5^{d}	1.9 ± 0.07^{b}	1.2 ± 0.4^{bc}	0.8 ± 0.1^{c}
mt	0.6 ± 0.05^{a}	0.5 ± 0.02^{a}	0.7 ± 0.5^{a}	0.5 ± 0.05^{a}	4.6 ± 0.8^{d}	1.9 ± 0.15^{b}	2 ± 0.6^{b}	1.3 ± 0.4^{b}
t_0 (day)								
wt	4.7 ± 1.2^{a}	3.8 ± 1.9^{a}	3.2 ± 0.3^{a}	3 ± 1^{a}	2.4 ± 0.4^{b}	2.3 ± 0.15^{b}	1.7 ± 0.3^{c}	2.6 ± 0.2^{b}
mt	3.2 ± 0.15^{a}	3 ± 1^{a}	3 ± 0.15^{a}	3 ± 1.2^{a}	2.8 ± 0.05^{b}	3.4 ± 2.1^{b}	2.3 ± 0.5^{b}	3.4 ± 0.9^{b}
S (%)								
wt	16 ± 22^{a}	85 ± 10^{b}	67 ± 22^{b}	65 ± 6^{b}	0 ± 0^{c}	3 ± 4.8^{c}	0 ± 0^{c}	0 ± 0^{c}
mt	19 ± 11^{a}	73 ± 12^{b}	67 ± 20^{b}	78 ± 13^{b}	1 ± 2.1^{c}	0 ± 0^{c}	0 ± 0^{c}	0 ± 0^{c}

Conidia were harvested from *CC* Colloidal chitin, *SPP Sphenarium purpurascens* particles, and *WB* wheat bran. k, Specific death rate, day^{-1}; t_0, delay time for starting death, day; S, survival at the end of the bioassay, %. Means labeled with the same letter were not statistically different. *NR* not reached, mortalities were below 40%

B. bassiana, Arcas et al. (1999) reported higher mortality for larvae of *Diatraea saccharalis* with conidia harvested from SDA medium added with yeast extract, compared to those values achieved by conidia harvested from WB. However, for another isolate in the same study, mortality was similar for conidia harvested from these two media. Significant observations in our study indicate that it is possible to choose a culture medium (SDA) as compared to others (CC, SPP, and WB) that induces a more virulent form of *B. bassiana* against larvae which in turn may help in the future to find the major causes of such virulence. Additionally, SDA was a more suitable medium for *mt* isolate since the highest conidial density was obtained, representing a 10-fold increase compared to the *wt* isolate, with the advantage that higher sporulation did not alter its virulence traits toward neither larvae nor adults of *T. molitor*.

Methodologically, the specific growth rate (μ) is a comprehensive physiological parameter useful to compare fungal growth patterns since this value takes into consideration mycelial branching frequencies in order to estimate biomass formation (Loera and Viniegra-González 1998); thus, it should be considered as suitable alternative to hyphal growth rate or U_r which is still widely reported (Liu et al. 2003; Ouedraogo et al. 1997). This technique showed that for the *mt* isolate, prefered substrates were WB and SSP, since higher μ values were a consequence of increased branching frequencies (shorter leading hyphal length).

Here we tested the importance of having two different isolates, a *wt* isolate and its 2-deoxy-glucose resistant mutant (*mt*) without previous characterization. These isolates showed measurable phenotypical differences according to culture media, including branching patterns related to hyphal length, germination rates, and conidial densities. However, there were no major differences in virulence when tested against either larval or adult insects of *T. molitor*.

As a final conclusion it seems to be of great importance to test differences between larval and adult stages of susceptible organisms for biocontrol purposes, as well as testing in each case the effect of the medium composition, which can change virulence patterns and conidial level production for each given isolate of the biocontrol pathogen. Such reproducible differences in virulence using different culture media may help to clarify the biochemical nature of such important biological features.

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