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Horizontal transfer and hypovirulence associated with double-stranded RNA in *Beauveria bassiana*

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ABSTRACT

Beauveria bassiana strains from different hosts and geographic origins were assayed for the presence of double-stranded RNA (dsRNA). Two of them (15.4 %) showed extra bands, with approximately 4.0–3.5 kb and 2–0.7 kb, respectively, after electrophoretic separation of undigested nucleic acids. Virus-like particles were approximately 28–30 nm diam. The dsRNA was maintained after conidiogenesis (vertical transmission) and was transmitted horizontally by hyphal anastomosis. Strains purged of dsRNA obtained after cycloheximide treatment showed increased conidial production when compared with strains carrying dsRNA particles. Bioassays demonstrated hypovirulence associated with dsRNA. The mean mortality against the insect *Euschistus heros* was reduced in strains containing dsRNA when compared with the isogenic dsRNA-free ones.

Introduction

Double-stranded RNA (dsRNA) viruses have been described in many fungal species. In most cases, these infections are latent and do not influence the host phenotype (Buck 1998). However, in some cases these particles are useful to the host, such as the killer system in *Saccharomyces cerevisae* (Wickner 1992) and *Ustilago maydis* (Shelbourn *et al.* 1988). Hypovirulence may also be associated with the presence of dsRNA as occurs in phytopathogenic fungi. dsRNA viruses were also described in entomopathogenic fungi such as *Metarhizium anisopliae* var. *anisopliae*, *M. flavoviride* (Leal *et al.* 1994; Bogo *et al.* 1996; Melzer & Bidochka 1998; Martins *et al.* 1999), *Beauveria bassiana* (Melzer & Bidochka 1998), and *Paecilomyces fumosoroseus* (Azevedo *et al.* 2000).

Beauveria bassiana is an important biocontrol agent and is pathogenic to several agricultural pests. The presence of dsRNA in *B. bassiana* was first reported by Melzer & Bidochka (1998) in two of 12 isolates analysed. There are no reports concerning hypovirulence related to the presence of dsRNA

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in this species nor is there any information about the transmission of dsRNA in this fungus. In this study, we report for the first time the horizontal transmission of dsRNA via parasexuality, and the elimination (cure) of dsRNA, as well as a dsRNA-associated hypovirulence, in *B. bassiana*.

Material and methods

Fungal strains

The Brazilian *Beauveria bassiana* strains used are listed in Table 1. Strains CG were provided by the Entomopathogenic Fungi Collection belonging to Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Recursos Genéticos (EMBRAPA-CENARGEN), Brasília, DF, Brazil. Strain E3 was provided by the I. C. Pimentel collection (Universidade Federal do Paraná, Brazil), and strain 252, by Sérgio Batista Alves collection (ESALQ, University of São Paulo, Piracicaba, São Paulo, Brazil). Strains 252J4/1 (with nutritional requirements for nicotinic acid and thiamine) and E3A1/1 (with nutritional requirements for biotin and cystein) were previouslly obtained by Dalzoto *et al.* (2003). Recombinants obtained via parasexuality from heterokaryons between 252J4/1 and E3A1/1 strains were also used (Table 2). The cultures are permanently stored in the fungal collection of the Laboratory of Microorganisms, Departamento de Genética, Universidade de São Paulo (ESALQ/USP), Piracicaba, Brazil.

Culture media and growth conditions

Liquid and solid complete (CM) and minimal medium (MM) were prepared as described by Pontecorvo *et al.* (1953). Incubation was performed at 28 °C for 14 d.

Nucleic acids extraction

Conidia from strains were inoculated at several points in a Petri dish containing CM, and after 4 d the colonies were removed carefully without the media and lyophilized. The colonies were ground to a fine powder using liquid nitrogen. Nucleic acids were extracted as described by Raeder & Broda (1985) and separated by electrophoresis in 1 % agarose gel stained with ethidium bromide (0.5 ng ml^{-1}) at 3 V cm^{-1}.

Table 1 – Strains of *Beauveria bassiana* used

Isolates	Insect host
E3	*Anthonomus grandis* (*Coleoptera*: *Curculionidae*)
CG10[a]	*Nezara viridula* (*Hemiptera*: *Pentatomidae*)
CG11 (Bb51)[a]	*Sternechus subsignatus* (*Coleoptera*, *Elateridae*)
CG12[a]	*Cycloneda sanguinea* (*Coleoptera*: *Coccinellidae*)
CG13 (Bb4)[a]	*Nezara viridula* (*Hemiptera*: *Pentatomidae*)
CG14 (BB44)[a]	*Podisus* sp (*Hemiptera*: *Pentatomidae*)
CG25[a]	*Anticarsia gemmatalis* (*Lepidoptera*: *Noctuidae*)
CG61[a]	*Diabrotica* sp. (*Coleoptera*:*Chrysomelidae*)
CG62[a]	*Diabrotica* sp. (*Coleoptera*:*Chrysomelidae*)
CG63[a]	*Forficula auricularia* (*Dermaptera*: *Forficulidae*)
CG66[a]	*Diabrotica* sp. (*Coleoptera*:*Chrysomelidae*)
E3A1/1[b]	
252J4/1[b]	

a Strains CG were provided by CENARGEN – EMBRAPA.
b Strains E3A1/1 (requiring biotin and cysteine) and 252J4/1 (requiring nicotinic acid and thiamine) are auxotrophic mutants from wild strains E3 and 252, respectively, as described by Dalzoto *et al.* (2003).

Table 2 – Recombinant strains of *Beauveria bassiana* from cross E3A1/1 (*bio cys*) × 252J4/1 (*nic thi*)

Recombinants	Requirements	Extra bands (dsRNA) size
C4R11	Bio, thi	+ (2 and 0.7 kb)
C4R12	Nic	+ (2 and 0.7 kb)
C4R13	Thi	–
C4R14	Bio, nic	–
C4R16	Cys	+ (2 and 0.7 kb)
C4R17	Thi	–
C4R18	Cys, nic	–
C4R19	Cys, thi	+ (2 and 0.7 kb)
C4R20	Nic	+ (2 and 0.7 kb)
C4R21	Cys	–
CRR22	Prototrophic	–

Nutritional requirements (bio, biotin; cys, cysteine; nic, nicotinic acid; thi, thiamine).
+ Presence; – absence.

Enzymatic digestions

Three digestions were performed using separately DNase I, RNase-A and S1 nuclease.as described by Bogo *et al.* (1996). For DNase digestion, the samples were treated with 70 U of enzyme (Gibco Life Technologies, Grand Island, NY) according to he supplier's instructions. For RNase-A digestion, the enzyme (Invitrogen, Carlsbad, CA) was added to 3 μg of total nucleic acids to a final concentration of 8 μg ml^{-1} and incubated at 37 °C for 1 h. Finally, for S1 nuclease digestion, 35 U enzyme (Gibco Life Technologies) were added per microgram of total nucleic acids and the reaction was performed according to the supplier's instructions.

Partial purification of virus-like particles

Partial purification was performed as described by Gillings *et al.* (1993) and modified by Howitt *et al.* (1995). Conidia were inoculated in flasks containing liquid CM under agitation (150 rev min^{-1}) at 28 °C for 48 h. Mycelia were harvested by filtration and washed three times with sterile water. Mycelium (10 g) was ground to a fine powder in liquid nitrogen. The powder was transferred to a 50 ml centrifuge tube, and 20 ml of cold 100 mM sodium phosphate buffer (pH 7.0) were added. The homogenate was extracted with 0.5 vol chloroform, centrifuged at 11 000 ***g*** for 20 min at 4 °C, and the upper aqueous phase transferred to 10 ml ultracentrifuge tubes. After ultracentrifugation at 120 000 ***g*** for 80 min at 4 °C, the resultant pellet was resuspended and maintained for 2 h in 1 ml 20 mM sodium phosphate buffer (pH 7.0). The tubes contents were stored at −20 °C overnight. Samples were clarified by centrifugation at 2000 ***g*** for 10 min at 4 °C, the supernatant made up to 10 ml with 20 mM sodium phosphate buffer and ultracentrifuged as above. The resulting pellets were resuspended in 0.5 ml of the same buffer for a minimum of 2 h and transferred to a 1.5 ml tube. Preparations were centrifuged at

1700 *g* for 5 min at 4 °C, and the supernatant retained. Samples were used directly or stored at 4 °C until examined by electron microscopy.

Electron microscopy

A 200-mesh parlodion/carbon grid was placed onto a 20 μl drop of the partly purified sample for 60 s and transferred to a droplet of stain for 30 s. Extracts were stained with 2 % uranyl acetate (pH 4.0). Grids were examined in a ZEISS EM 900 at 80 KV, and the images were processed by the Kontron Eletronik KS 300 system.

Heterokaryon formation and horizontal transmission tests

Heterokaryons from mutant strains 252J4/1 and E3A1/1 were obtained using the process described by Azevedo & Roper (1970), and the recombinant products, with auxotrophic markers differing from parental types, were analysed for the presence of dsRNA. Nucleic acids were purified (Raeder & Broda 1985) and separated by electrophoresis in 1 % agarose gel stained with ethidium bromide (0.5 ng ml^{-1}) at 3 V cm^{-1}. A RAPD analysis was performed (as described below) to provide identification of the initial strains after a horizontal transmission test.

RAPD analysis

Analysis of RAPD was carried out as described by Dalzoto *et al.* (2003) in 25 μl final volume, containing 45–55 ng DNA template, 3 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Life Technologies, Alameda, CA), 0.4 mM primer (OPX1, 5′-CTGGG CACGA-3′; OPX6, 5′-ACGCCAGAGG-3′; OPX7, 5′-GAGCGAGG CT-3′; Operon Technologies), 0.2 mM of each dNTP in 20 mM pH 8.4 Tris–HCl containing 50 mM KCl. PCR amplifications were conducted with an initial denaturation at 92 °C for 4 min, which was followed by 40 PCR amplification cycles consisting of denaturing at 92 °C for 1 min, annealing at 37 °C for 1.5 min, and extension at 72 °C for 2 min followed by a final extension of 5 min at 72 °C. The amplification products were separated by electrophoresis on 1.5 % agarose gels and visualized by ethidium bromide staining. All reactions were repeated at least twice, and negative controls containing water instead of DNA were also used.

Curing experiments

A single-conidium subculture from strain CG25 was inoculated at 15 points in a Petri dish containing CM and the plate incubated for 14 d at 28 °C. The colonies obtained were transferred to dishes containing cycloheximide (20 μg ml^{-1}) and further incubated for 14 d. Colonies were then transferred to dishes containing CM and incubated for 10 d. After this period, nucleic acids were purified and separated by electrophoresis as already described. As a control to show the stability of dsRNA in the absence of cycloheximide, a conidial suspension of strain CG25 was inoculated in dishes containing CM. After 7 d, ten single-conidium subcultures were inoculated in CM and incubated for 14 d at 28 °C. Conidia from each subculture were inoculated at several points on a plate containing CM and after 3 d of growth were collected and lyophilized. The purification and separation of nucleic acids was performed as reviously described. As a control for the possibility that the cured strains recovered were contaminants, an RAPD analysis was performed using the three primers already described.

Determination of growth and conidiation

Conidia from isogenic strains with and without dsRNA were inoculated in the centre of CM dishes and incubated for 14 d at which point the diameter of colonies was measured. Also after that time, 10 ml of a Tween 80 (0.1 % v/v) aqueous solution were added to each plate, and the conidia dislodged with a glass spreader. The suspension was adjusted to 10 ml with Tween 80 solution, vortexed to disperse the conidia, and the conidial concentration was estimated with the aid of a Neubauer chamber. This procedure was repeated 18 times, the data were transformed to $\log(x+2)$ and analysed by analysis of variance (ANOVA; NCSS, Kaysville, Utah).

Virulence tests

Conidia suspensions of each strain analysed (10 μl of a suspension of 10^7 conidia ml^{-1} sterile water per insect) were applied on the ventral region (abdomen) of ten adult *Euschistus heros* (*Hemiptera*: *Pentatomidae*) insects per treatment. The insects provided by the Department of Entomology, Universidade Federal do Paraná, Brazil, were maintained in sterile glass flasks, covered with sterile gauze and fed *Legustrum lecanum*, soybean and peanuts. Cotton soaked in sterile water was placed above the gauze to provide water to insects. The experiment was maintained under laboratory conditions (28 °C, 12 h light regime and 55–66 % RH) and repeated four times. The search for dead insects started 24 h after inoculation. Mortality data were modified to $\log(x+2)$ and submitted to ANOVA. Dead bodies were incubated in sterile Petri dishes containing filter paper soaked in sterile water, at 28 °C, until fungi emergence. Fungi that emerged from dead bodies were isolated, their nucleic acids were purified and separated by electrophoresis as previously described. A RAPD analysis was performed to confirm the identity of the isolates from each treatment.

Results

Presence and characterization of dsRNA

Among 13 *Beauveria bassiana* strains analysed, two of them (15.4 %) showed bands, of approximately 4.0 and 3.5 kb for strain CG25 (Fig 1) and 2.0 and 0.7 kb for strain 252J4/1, (data not shown) indicators of the presence of dsRNA. This was confirmed by digestion of total nucleic acid preparation separately with DNase and RNase A showing that these bands were sensitive only to RNase A. These bands were also resistant to digestion with S1 nuclease in concentrations higher than 35 U at 37 °C for 40 min. The following analysis was performed only in strain CG25 as strain 252J4/1 is an auxotrophic mutant and this could harm the results related to virulence tests.

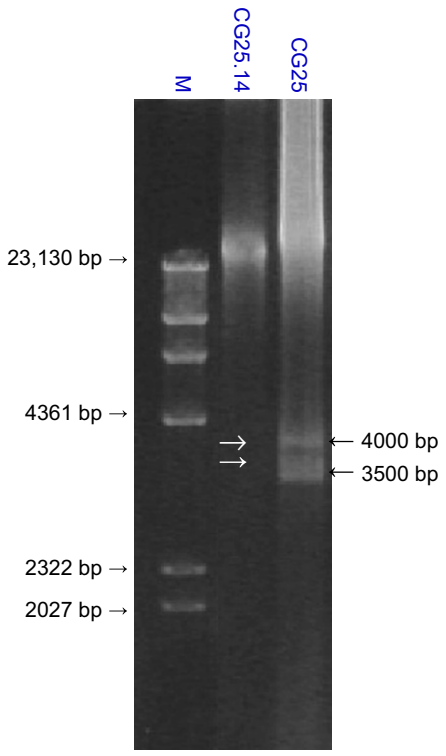

Fig 1 – Double-stranded RNA in *Beauveria bassiana* isolates M: λ DNA digested with *Hind*III (molecular weights at left; Life Technologies). CG25.14: *B. bassiana* strain CG25 dsRNA free. CG25: *B. bassiana* strain CG25 with dsRNA. Arrows indicating dsRNA bands (approximated molecular weights at right).

Electron microscopy

The presence of dsRNA in fungal cells suggested infection by mycoviruses and led to the analysis of virus-like particles (VLPs) by TEM. VLPs approximately 28–30 nm in diameter were found in strain CG25 of *Beauveria bassiana* (Fig 2).

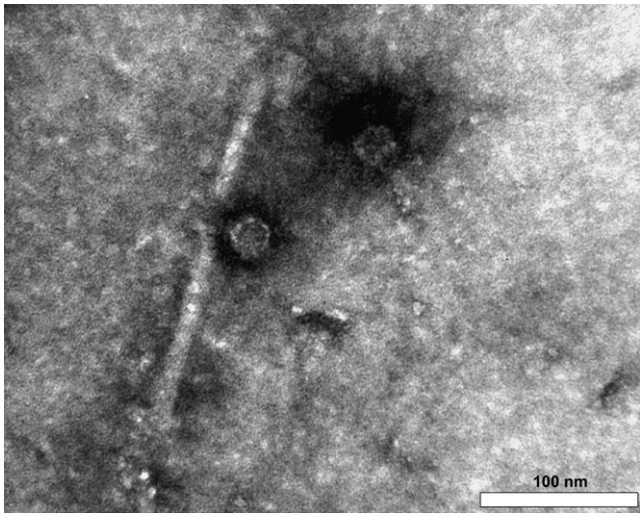

Fig 2 – Virus-like particles in *Beauveria bassiana* strain CG25. Bar = 100nm.

Horizontal transmission of dsRNA

Heterokaryons formed between auxotrophic mutant strains E3A1/1 (dsRNA-free) and 252J4/1 (dsRNA-containing) produced recombinants by a parasexual process already described for *Beauveria bassiana* by Paccola-Meirelles & Azevedo (1991, 1994). Some recombinant products presented the extra bands of dsRNA (Table 2) indicating that horizontal transmission of dsRNA is occurring in *Beauveria bassiana*. RAPD markers from these recombinants are identical to parental 252J4/1.

Curing experiments

Ten single-conidial subcultures from strain CG25 were assayed for the presence of dsRNA, and all of them showed extra bands (data not shown). This result suggests that dsRNAs in *Beauveria bassiana* are stable, even after, at least 100 mitotic divisions, when cycloheximide is not present in culture media. Treatment with cycloheximide was efficient to obtain dsRNA-free colonies from strain CG25 of *Beauveria bassiana*. Identical marker patterns were found by RAPD analysis, both in original and cured strains.

Growth and conidiation associated to dsRNA

Differences between isogenic strains with and without dsRNA were observed to be related to conidial production: strain CG25 produced significantly fewer conidia than its isogenic strain without dsRNA. Conversely, radial colonial growth did not show statistical differences between infested and virus-free isogenic strains (Table 3).

Virulence tests

Results of bioassays against *Euschistus heros* using isogenic strains of *Beauveria bassiana*, with and without dsRNA showed statistical differences between them when mean mortality after treatment was compared. These differences occurred after 5 and 7 d inoculation but not after 9 d post-inoculation (Fig 3).

Fungi emerging from *E. heros* dead bodies after treatments were isolated. Their nucleic acids were purified, and their RAPD marker patterns observed were the same as the original strains involved in the treatments, thus demonstrating that the insects were killed by the inoculated fungi and not by a previous infection.

Table 3 – Diameter of colonies (cm) and conidial production in *Beauveria bassiana* isogenic strains growing for 14 d on CM

	Colony diam (cm)	Conidial production ($\times 10^6$ ml^{-1})
CG25 (+dsRNA)	5.29 ± 0.36 a	7.34 ± 4.07 a
CG25.14 (−dsRNA)	5.19 ± 0.16 a	18.71 ± 6.30 b

Results showing identical letters have no statistical differences at 5 % level.

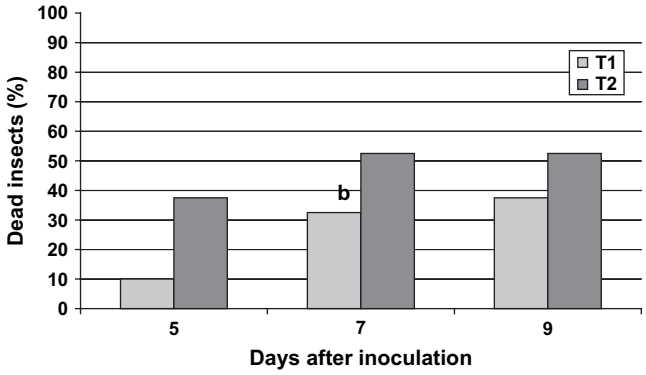

Fig 3 – Mortality of *Euschistus heros* submitted to isogenic strains of *Beauveria bassiana*. T1(treatment 1) *B. bassiana* strain CG25 with dsRNA; T2(treatment 2) *B. bassiana* strain CG25.14 dsRNA free. Different letters imply statistical differences among strains at the 5 % level.

Discussion

We found dsRNA in 15.4 % strains of *Beauveria bassiana* analysed. In *B. bassiana*, the presence of dsRNA was reported by Melzer & Bidochka (1998), in two of 12 isolates analysed, showing two different band patterns of 3 and 2 kb. In phytopathogenic fungi, dsRNAs with sizes ranging between 800 bp and 15 kb were found (Howitt *et al.* 1995; Castro *et al.* 1999; Preisig *et al.* 2000; Papp *et al.* 2001; Robinson & Deacon 2002). The presence of dsRNA in fungal cells strongly suggests infection by mycoviruses, and when these infections show multiple dsRNA segments, which commonly occurs this could be related to infection by mycoviruses with segmented genomes (Buck 1986).

The presence of VLPs was confirmed by electron microscopy in strain CG25 (Fig 2). Those bands may result from multiple infection and not from a single RNA virus with a segmented genome. Bogo *et al.* (1996) detected dsRNA in *Metarhizium anisopliae* and found virus-like particles 35 nm diam, while Leal *et al.* (1994) described 33 nm particles. In phytopathogenic and endophytic fungi, 28–50 nm particles can be found (Hunst *et al.* 1986; Howitt *et al.* 1995; Castro *et al.* 1999; Zabalgogeazcoa *et al.* 1998; Papp *et al.* 2001). The size of VLPs found in this work agrees with the range found in many others fungi. However, not all the dsRNA fragments are encapsidated. These uncapsidated elements are packaged in lipid membrane vesicles and have been described in such fungi as *Cryphonectria parasitica* (Hansen *et al.* 1985), *Rhizopus* (Papp *et al.* 2001), and *Epichloë festucae* (Zabalgogeazcoa *et al.* 1998).

There are no known natural vectors that transmit dsRNA viruses, although the transmission of these viruses occurs vertically through cell division and conidial production or horizontally by cell fusion between genetically vegetative compatible strains (Buck 1998). Horizontal transmission through hyphal anastomosis was observed in *Aspergillus* spp. (Varga *et al.* 1994), *Nectria radicicola* (Pyung & Hwan 2001) and *Diaporthe ambigua* (Preisig *et al.* 2000). Attempts at horizontal transmission failed in *E. festucae* (Zabalgogeazcoa *et al.* 1998), *Pythium irregulare* (Gillings *et al.* 1993), and *M. anisopliae* (Bogo *et al.* 1996).

As already shown (Dalzoto *et al.* 2003) recombination in *B. bassiana* is characterized by an intra-hyphal transformation where one parental genome is a receptor for DNA from another parental genome. Cell lysis, which is induced when hyphal fusions occur between strains with heterokaryon incompatibility in filamentous fungi (Glass *et al.* 2000), may release genetic material resulting in intra-hyphal transformation. Similar findings have been reported in some other fungi, with Stasz & Harman (1990) and Harman *et al.* (1998) coining the term 'interstrain gene transfer' to explain the process by which some *Trichoderma* recombinants were formed during protoplast fusion. These authors suggest that there were two types of strains, non-prevalent and prevalent, and that some of the nuclei from the non-prevalent strain are degraded and that genetic material is inserted into the genome of the prevalent strain to produce recombinants without altering the RAPD banding pattern. Viaud *et al.* (1996) also recovered recombinants with the profile of only one of the parental strains while investigating the protoplast fusion products of two *Beauveria* species. The presence of dsRNA in recombinant products obtained by parameiosis in *B. bassiana* can be considered another marker indicating which parental strain is the prevalent one and corroborates the hypothesis of intra-hyphae transformation.

The persistence of dsRNA extra bands in subcultures of strain CG25, even after at least 100 mitotic divisions suggests that dsRNAs in *B. bassiana* are stable when cycloheximide is not present in culture media.

The curing mechanism is still unknown, but it has been recorded that cycloheximide inhibits RNA synthesis (Bottacin *et al.* 1994; Elias & Cotty 1996). Azevedo *et al.* (2000) obtained dsRNA-free cultures in *Paecilomyces fumosoroseus* by single-conidial subculture. Melzer & Bidochka (1998) obtained a cured strain of *M. anisopliae* by treatment with cycloheximide, while Martins *et al.* (1999) did not obtain any cured strains of *M. flavoviride* either after treatment with cycloheximide, by single-conidial subcultures, or by elevated incubation temperature. Many treatments are described to obtain free dsRNA cultures, e.g., chlorate selection of *nit* mutants exposed to hot water exposure, antibiotics, hyphal tip transfer, ultraviolet radiation (Pusey & Wilson 1982; Fulbright 1984; Hunst *et al.* 1986; Kousik *et al.* 1994; Elias & Cotty 1996) and can be successful or not in many fungi. In fungi that have multiple dsRNA segments the cure is frequently partial and the smaller fragments are lost (Robinson & Deacon 2002; Ikeda *et al.* 2004). The possibility of dsRNA elimination by an RNA synthesis inhibitor such as cycloheximide may be important in future pathogenicity studies to improve strains for the biological control of agricultural pests.

Differences were related to conidial production. Strain CG25 produced significantly fewer conidia than its isogenic strain without dsRNA (Table 3), as was also reported in other fungi such as *B. cinerea* (Howitt *et al.* 1995), *E. festucae* (Zabalgogeazcoa *et al.* 1998), *Flammulina velutipes* (Magae & Hayashi 1999), *Aspergillus* spp. (Varga *et al.* 1994) and *Rhizopus* (Papp *et al.* 2001). However, morphological changes, such as altered colony morphology have been described in several fungi

associated with the presence of dsRNA, as in *Chalara elegans* (Punja 1995), *M. anisopliae* (Melzer & Bidochka 1998; Gimenez-Pecci *et al.* 2002), *D. ambigua* (Preisig *et al.* 2000), and *Nectria radicicola* (Pyung & Hwan 2001). Tiago *et al.* (2004) detected a reduction in conidial production in *M. anisopliae* var. *acridum* associated with the presence of dsRNA.

Virulence change associated with absence of dsRNA was detected at least until 7 d after inoculation suggesting that dsRNA free strain is able kill the insects more rapidly when compared with the dsRNA-containing strain (Fig 3). Hypovirulence associated with dsRNA has been observed in many phytopathogenic fungi such as *Cryphonectria* (Elliston 1982), *Botrytis cinerea* (Castro *et al.* 1999; 2003), *Diaporthe ambigua* (Preisig *et al.* 2000), *Fusarium graminearum* (Chu *et al.* 2002) and *Sclerotinia* (Bolland 2004). No differences in pathogenicity have been observed in dsRNA-containing and dsRNA-free entomopathogenic fungi as reported in *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *acridum* and *M. flavoviride* (Leal *et al.* 1994; Bogo *et al.* 1996; Martins *et al.* 1999; Tiago *et al.* 2004). Conversely, Melzer & Bidochka (1998) described increased virulence in dsRNA-free strains of *M. anisopliae* compared with isogenic strains with dsRNA as was also reported for *P. fumosoroseus* (Azevedo *et al.* 2000). The results presented here seem to be the first example of change of virulence in *B. bassiana* dsRNA-free strains compared with isogenic strains containing dsRNA in *B. bassiana*.

These results suggest that dsRNAs in *B. bassiana* are responsible for the hypovirulent effect against *E. heros*, in a manner similar to that observed in several other phytopathogenic fungi, and it seems that the decrease in conidial production is important to establish hypovirulence in *B. bassiana*. Although the differences in pathogenicity between dsRNA-free strain and dsRNA-containing strain were statistically significant, during at least one week period after the insect inoculations, the biological meaning of dsRNA in entomopathogenic fungi remains uncertain.

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