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Intra-specific Variation in Virulence and *In Vitro* Production of Macromolecular Toxins Active Against Locust Among *Beauveria bassiana* Strains and Effects of *In Vivo* and *In Vitro* Passage on These factors

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Strains of Beauveria bassiana *isolated from locust or from the soil varied considerably in their virulence and their ability to produce* in vitro *toxic metabolites against* Locusta migratoria. *Among the pathogenic isolates, only culture filtrates of 90/2-Dm, 92/11-Dm and 0023-Su were toxic by injection, a result which demonstrates that isolates of* B. bassiana *can be pathogenic for* L. migratoria *whether they secrete toxic metabolites* in vitro *or not. Toxic metabolites secreted by strains 90/2-Dm and 92/11-Dm were macromoleculer as they were retained by dialysis (cut-off of 6–8 kDa for globular proteins), whereas those secreted by 0023-Su were not. The effect o f* in vitro *passage on virulence and on toxicogenic activity of isolate 90/2-Dm was dependent on the mycological media the inoculum was produced on. The virulence of isolate 90/2-Dm was significantly reduced after two passages through Sabouraud Dextrose Agar (SDA) whereas two passages through Malt Agar (MA) increased its virulence and its toxicogenic activity. Nevertheless, the most aggressive conidia and the most toxic macromolecules were obtained after two passages of the isolate 90/2-Dm through the host. The bioactive macromolecules present in the crude filtrate of isolate 90/2-Dm were precipitated by 90% saturation of ammonium sulphate, and the insecticidal activity was exclusively detected in high molecular mass fraction after gel filtration on Sephadex G-25. In addition, the insecticidal effect of the Sephadex G-25 fraction was significantly reduced after exposure for 2 h at 60°C and 20 min at 120°C, suggesting that the insecticidal metabolites in the culture filtrates of isolate 90/2-Dm were proteinaceous.*

Keywords: *entomogenous fungi,* Beauveria bassiana*, biological control, locust,* Dociostaurus maroccanus*,* Locusta migratoria*, toxic metabolites, macromolecular toxins, bioinsecticides, insecticidal effects*

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INTRODUCTION

The exploitation of entomopathogenic fungi for biological control of locust and grasshoppers has received much attention, especially in recent years (Prior & Greathead, 1989; Mason & Erlandson, 1994). That is true for the Moroccan locust *Dociostaurus maroccanus* (Thunberg) widely distributed in the Mediterranean area where it is an important pest (Vázquez-Lesmes & Santiago-Álvarez, 1993). The preventive control of *D. maroccanus* relies on broad-spectrum synthetic insecticides (Latchininsky & Launois-Luong, 1992). This is likely to change because some environmental issues arising from their use such as impact on operators, other people, livestock, and other arthropods, including natural enemies of this locust, have increased the demand for biological control (Lomer *et al*., 2001; Quesada-Moraga & Santiago-Álvarez, 2001).

Studies on natural enemies of orthopteran insects have shown that the fungus *Beauveria bassiana* (Bals.) Vuill. causes epizootics in *D. maroccanus* populations (Hernández-Crespo & Santiago-Álvarez, 1997), and is therefore considered, together with *Bacillus thuringiensis* (Berliner) (Quesada-Moraga & Santiago-Álvarez, 2001) as a main candidate for the biological control of the Moroccan locust (Jiménez-Medina *et al.*, 1998). Furthermore, natural isolates of this hyphomycete exhibit a high insecticidal activity when tested against *D. maroccanus* in laboratory assays (Jiménez-Medina *et al*., 1998). In field tests, the most virulent strain was more effective when applied as bait than by spraying (Jiménez-Medina *et al*., 1996).

Field assays with fungi have often produced inconsistent results. In addition, their slow mode of action compared with chemical insecticides has hindered commercial development (Butt *et al*., 2001). Therefore, to improve commercialibility of a fungus as a biological control agent, its performance must be improved in most cases. This may be achieved by incorporating more toxic modes of action to increase killing rates (St Leger *et al*., 1996; St Leger & Screen, 2001). For this aim, the knowledge of the mode of action of entomopathogenic fungi is vital to develop effective commercial mycoinsecticides.

It has been shown that many entomopathogenic fungi including *B. bassiana* kill their hosts after only limited growth in the hemocoel, and that toxins are probably responsible for host death (Roberts, 1981). Furthermore, the penetration and development of *B. bassiana* in susceptible hosts involves the secretion of diverse toxic secondary metabolites. Among them are well-characterised low molecular weight insecticidal cyclic peptides (Roberts, 1981; Khachatourians, 1991; Vey *et al*., 2001) and poorly studied macromoleculer insecticidal metabolites, which have been directly implicated in the insecticidal properties of this hyphomycete (Mazet *et al*., 1994). Unfortunately, there are only a few studies on the mode of action of *B. bassiana* on locusts, and hardly any on the production of toxic macromolecules during infection of these hosts (Vey *et al*., 2001). Information is highly desirable because the selection of strains with high toxin production may eventually help identify particularly potent *B. bassiana* strains for the control of *D. maroccanus.*

For the use of entomopathogenic fungi in biological control, not only their virulence is important but also possible variations between batches of fungal propagules, and issues concerning the preservation of viability in biopreparations. More precisely, commercially available biocontrol agents have to be produced in large quantities by means of large-scale fermentation. But the continual production of inoculum on artificial media may lead to a loss of virulence of the organism (Wraight *et al*., 2001). In fact, throughout the literature on the entomopathogenic fungi, the effect on virulence of repeated subculturing in mycological media or passage through insect hosts has been reported by different authors (Kawakami, 1960; Schaerffenberg, 1964; Ferron *et al*., 1972; Fargues & Robert, 1983; Brownbridge *et al*., 2001). In contrast, no reports are available on the effect of passage of conidia through mycological media and through the host on the production of toxic metabolites by the pathogen.

In this work, an attempt has been made to determine whether a correlation exists between the *in vitro* production of toxic metabolites and the pathogenicity for *Locusta migratoria* L. for a range of *B. bassiana* isolates. We selected *L. migratoria* as a model insect host because it is easily reared at controlled conditions. One of the fungal strains secreting toxic macromolecules *in vitro* was chosen for a more detailed analysis of such macromolecules and to study whether successive passages through the host and through mycological media would affect its virulence and its toxicogenic activity.

MATERIAL AND METHODS

Fungi and Insects

The strains used in this study were obtained from Dr. Cándido Santiago-Álvarez, University of Córdoba (Spain). Strains EABb 90/2-Dm, EABb 91/7-Dm, EABb 92/10-Dm and EABb 92/11-Dm were isolated from *D. maroccanus* (Orthoptera: Acrididae) in the breeding area of 'la Serena' in Badajoz (Spain) in 1990, 1991, 1992 and 1992, respectively. Strain EABb 91/6-Ci was isolated from the Italian locust *Calliptamus italicus* L. in the same breeding area in 1991. Finally, the soil strains EABb 00/23-Su and EABb 00/26-Su were isolated respectively from El Hierro (Canary Islands) and from 'la Serena' in 2000. Slant multispore cultures of strains were grown on Malt Agar (MA Biokar Diagnostic) at 25°C in the dark and then stored at 4°C. Conidial suspensions were prepared by scraping conidia from well sporulated 21-day-old slant cultures into sterile distilled water, and estimating their concentration using a hemocytometer. Viability of conidia was checked before preparation of suspensions by germinating tests in liquid Czapek-Dox broth plus 1% (w/v) yeast extract medium. In all the experiments, germination rates were higher than 90%.

Newly emerged fourth instar *L. migratoria* nymphs (mean weight 361 ± 31 mg) from a long established healthy colony at the Station de Recherches de Pathologic Comparée, INRA-CNRS, Saint-Christol-Lez-Alès, France, were used as a host. New locusts are regularly introduced to this colony to maintain the vigour of the insects.

Experimental Infections

To assess the pathogenicity of each strain, 8 μL of a spore suspension adjusted to 1×10^5 conidia mL^{-1} were injected through the intersegmental membrane between the second and third abdominal segments, using a Desaga microinjector. Control nymphs were injected with the same volume of sterile distilled water. Dead insects were removed and placed in a humid chamber (100% RH) at 30°C to encourage sporulation.

Batches of 10 nymphs, maintained in Plexiglas-framed cages ($12 \times 12 \times 17$ cm) with mosquito-mesh sides and roof, were placed in incubators at 28°C, 60% relative humidity and a 16:8-h L:D photoperiod. Each treatment was replicated four times with 10 nymphs/replicate and the whole experiment was repeated. All locusts were fed fresh wheat seedlings. Nymphal mortality was recorded every 24 h, within 10 days postinjection.

Preparation of Crude Filtrates

To prepare a primary culture, 1 mL of a suspension of conidia (first adjusted to 1×10^7 conidia ml^{-1}) was inoculated into 25 mL of Adamek's liquid medium (40 g glucose; 40 g yeast extract; 30 g corn steep liquor (Sigma); 1000 mL distilled water) in a 100-mL Erlenmeyer flask and cultured at 25°C on a rotatory shaker (CFL 1092) at 110 r.p.m. for 4 days. To inoculate secondary cultures for large-scale growth of the fungus, 2 mL of the primary culture were transferred into 250 mL of the same medium in a 1-L Erlenmeyer flask, and cultured under the same conditions on a TR 125 INFORS shaker for 7 days, before removing the mycelial material by filtration through filter paper (Whatman No. 3chr).

Dialysis

One volume of unconcentrated crude filtrate was dialysed against two volumes of distilled water for 24 h at 4°C. The sample was introduced in a molecular porous membrane (Spectra) with a cut-off of 6–8 kDa for globular proteins. The adialysates were concentrated at 4°C by embedding the membrane in polyethylene glycol 20000 (Fluka), while the dialysates were concentrated by evaporation at room temperature in a chemistry hood. 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.

Passage of the Selected Strain through Mycological Media and through the Host

To study the effect of *in vitro* passage through mycological media, the selected strain was sequentially subcultured by mixed conidial transfer to full-strength MA and Sabouraud Dextrose Agar (SDA, Oxoid). Each culture corresponding to a passage was incubated in the dark at 28°C for 3 weeks before the next subculture. Conidia were harvested from the second series of subcultures by scraping the conidia from the surface of 21 day-old cultures and suspensions were used to assess either virulence or toxin production *in vitro* as previously described.

To study the effect of passage through the host, 8 μL of a spore suspension adjusted to 1×10^5 conidia ml^{-1} were injected into 20 fourth instar nymphs of *L. migratoria*. Dead insects were collected and placed under humid conditions (100% RH) at 30°C to stimulate sporulation, and conidia sampled at the surface of sporulating cadavers were used to pass the fungus two times through the host. Finally, suspensions of conidia isolated from a sporulating cadaver of the second passage were used either to assess virulence or toxin production *in vitro* as previously described.

The conidia obtained from a stock slant culture, and after two passages through either MA, SDA or the host were designated Cl, C2, C3, and C4, respectively. These four types of conidia were used to inoculate different cultures from which crude filtrates F1, F2, F3, and F4 were obtained, respectively. Adialysates Al, A2, A3 and A4 and dialysates Dl, D2, D3, and D4 were prepared as described above by dialysis of crude filtrates F1, F2, F3, and F4, respectively.

Toxicity of the Sources of Fungal Molecules

To assess the toxicity of filtrates, adialysates and dialysates, 8 μL of each solution were injected as described above, after sterilisation through a 0.2-μm Dynagard filter unit. Control nymphs were injected with either sterile uninoculated broth or with fractions resulting from its dialysis. Each treatment was replicated four times, 10 nymphs each, and the whole experiment was repeated. Nymphal mortality was monitored every 24 h, within 10 days postinjection. Observations were also made on development and feeding behaviour of the insects. Dead insects were dissected under a binocular microscope to observe the signs of activity of the injected molecules on insect tissues. Observations on the presence of any chromatic changes or morphological alterations were made on tracheae, air sacs, digestive track, fat body, muscle, cuticule and genitalia. Samples of tissues showing the signs of activity were also examined with a Nikon Microphot-FXA optic microscope. The experimental conditions of these assays were the same as described for the experimental infections.

The effect of passages through the host and through mycological media on virulence and toxin production of the selected strain was assessed and monitored following the same procedure. Filtrates F1, F2, F3 and F4, and adialysates A1, A2, A3 and A4 were injected as described above, and insects injected with either sterile uninoculated broth or with fractions resulting from its dialysis were used as controls. Each treatment was replicated four times, 10 nymphs each, and the whole experiment was repeated twice.

Precipitation of the Crude Filtrate of the Strain 90/2-Dm by Ammonium Sulphate

Proteins from the filtrate of 90/2-Dm, precipitated with 90% of saturation of ammonium sulphate, were collected by centrifugation at $10\,000 \times g$ for 30 min. The precipitate was desalted through a Sephadex G-25 (Pharmacia) column (2.5×30 cm) in 50 mM Tris–HCl buffer, pH 8.0. Then, the desalted fraction was injected to fourth instar *L. migratoria* nymphs as described above. 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. Each assay was replicated four times, 10 nymphs each, and the whole experiment repeated twice. Dead insects were dissected under a binocular microscope to observe the signs of activity of the injected molecules on insect tissues. Observations on the presence of any chromatic changes or morphological alterations were made on tracheae, air sacs, digestive track, fat body, muscle, cuticule and genitalia. Samples of tissues showing the signs of activity were also examined with a Nikon Microphot-FXA optic microscope. The experimental conditions of these assays were the same as described for the experimental infections.

Temperature Effects

Desalted fractions collected on Sephadex G-25 were incubated 2 h at 60°C and 20 min at 120°C, and then injected into fourth instar *L. migratoria* nymphs. 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. Each assay was replicated four times, 10 nymphs each, and the whole experiment repeated.

Statistical Analysis

Mortality data were analyzed with one-way analysis of variance (ANOVA) and the least significant difference (LSD) test was used to compare means in Statistic for Windows (Anonymous, 1996). In Table 1, percentage mortality caused by crude filtrates, adialysates and dialysates has been corrected for mortality in the controls using Abbott's formula (Abbott, 1925). The cumulative mortality response across the assessment period was analysed with Kaplan–Meier survival analysis in SPSS (Statistical Package for Social Sciences in personal computers) 8.0 for Windows (SPSS, 1997). LT_{50} values were calculated by mortality versus time regressions by Probit analysis. These regressions were also performed using SPSS 8.0 program.

RESULTS

Pathogenicity and *in vitro* Production of Metabolites Toxic for *L. migratoria* of *B. bassiana* Isolates

There was intra-specific variation in pathogenicity and *in vitro* secretion of toxic metabolites among the seven strains of *B. bassiana* tested (Table 1). All isolates were pathogenic for *L. migratoria* causing 57.5–94.4% mortalities within 10 days, with the exception of strain 92/10-Dm, which was non-pathogenic at a dose of 8×10^2 conidia per insect. Significant differences were detected between strains ($F7{,}56 = 282.1$, $P < 0.001$ for 8×10^2 conidia per insect). The most virulent strains were 0023-Su and 0026-Su causing over 90% mortality, followed by 91/7-Dm which caused 78.8% mortality, and by the rest of strains which caused mortalities ranging between 57.5 and 61.9% (Table 1). The LT_{50} values of each strain infecting locusts differed significantly (confidence limits non-overlapped). The lowest LT_{50} value was 3.4 days for isolate 0023-Su, followed by 4.4 days for 0026-Su and 5.4 days for 91/7-Dm. No significant difference was detected between LT_{50} values of isolates 90/2-Dm (6.8 days), 91/6-Ci (6.9 days) and 92/11-Dm (7.1 days).

There were also significant differences between isolates in toxicity of crude filtrates ($F_{7,56} = 170.11$, $P < 0.001$ for 4.8 µg of total protein/insect) and adialysates ($F_{7,56} = 196.72$,

TABLE 1. Susceptibility of fourth instar nymphs of *L. migratoria* to different strains of *B. bassiana* and to different sources prepared from their cultures in Adamek's liquid medium. Nymphs inoculated respectively by injection of 8×10^2 conidia per insect, 8 μL of crude filtrates at a dose of 600 μg of total protein ml, 8 μL of adialysates at a dose of 900 μg of total protein of ml and 8 μl of dialysates. Four replicates with 10 nymphs/replicate and whole experiment repeated two times

	Conidia		Crude filtrate		Adialysatet		Dialysate	
	Mean mortality (%±SD)*	LT_{50} (95% conf int)**	Mean mortality (%±SD)	LT_{50} (95% conf int)	Mean mortality (%±SD)	LT_{50} (95% conf int)	Mean mortality (%±SD)	LT_{50} (95% conf int)
EABb 90/2-Dm	57.5±9.3 a	6.8 (5.9–7.9)	56.3±4.4 a	8.0 (7.4–8.8)	60.0±7.5 a	6.1 (5.2–6.9)	4.2±1.8 a	DA
EABb 91/6-Ci	61.3±5.8 a	6.9 (6.0–8.1)	14.4±3.2 b	DA	8.1±2.5 b	DA	5.6±3.2 a	DA
EABb 91/7-Dm	78.8±6.9 b	5.4 (4.6–6.2)	11.3±3.5 bc	DA	8.8±4.3 b	DA	6.3±2.3 a	DA
EABb 92/10-Dm	0.0 c	DA	11.3±2.3 bc	DA	11.2±3.5 b	DA	5.0±2.6 a	DA
EABb 92/11-Dm	61.9±5.9 a	7.0 (6.2–8.1)	48.8±6.9 d	DA	52.5±5.9c	7.2 (6.4–8.1)	4.3±1.7 a	DA
EABb 0023-Su	94.4±4.9 d	3.4 (2.8–4.4)	35.0±5.3 e	DA	10.0±2.6 b	DA	10.6±4.9 b	DA
EABb 0026-Su	90.0±7.5 d	4.3 (3.8–5.0)	10.6±3.2 bc	DA	12.5±1.5 b	DA	5.0±3.8 a	DA
Control	5.6±1.7 e	DA	7.5±2.6 c	DA	6.8±2.5 b	DA	4.5± 2.8 a	DA

*Mortality by injection after 10 days post injection. Means within columns with the same letter are not significantly different (least significant difference $P > 0.05$). Mortality caused by crude filtrates, adialysates and dialysates corrected using Abbott's formula.

**LT_{50} times in days when 50% of nymphs were dead.

DA, insects were not killed and ultimately developed into healthy adults.

$P < 0.001$ for 7.2 μg of total protein/insect). Significant insecticidal activity was only detected when nymphs were injected with crude filtrates of isolates 90/2-Dm, 92/11-Dm, 0023-Su and 91/6-Ci (Table 1). Crude filtrate of 90/2-Dm was the most toxic one, causing 56.3% mortality, followed by 92/11-Dm, causing 48.8% mortality and by 0023-Su, with caused 35.0% mortality. Mortality caused by crude filtrate of 91/6-Ci was also slightly higher than controls (Table 1). In contrast to crude filtrates, significant insecticidal activity was only detected when nymphs were injected with adialysates of isolates 90/2-Dm and 92/11-Dm, adialysates of strains 0023-Su and 91/6-Ci being non-toxic (Table 1). The adialysate of isolate 90/2-Dm was the most toxic one, causing 60% mortality, followed by adialysate of 92/11-Dm, which caused 52.5% mortality. The adialysate of isolate 90/2-Dm had a LT_{50} of 6.1 days, which was significantly lower than that of 7.2 days of isolate 92/11-Dm (Table 1). Injection of dialysates of each strain caused no significant mortality, with the exception of isolate 0023-Su, which caused a weak, however significant mortality of 10.6%.

Effect of Passage through Mycological Media and Through the Host on Virulence and Secretion of Toxic Metabolites of Strain 90/2-Dm

The effect of the *in vitro* passage of the strain 90/2-Dm on its virulence was dependent on the mycological media the inoculum was produced on. Significant differences were detected between average survival time (AST) of controls and of insects injected with conidia Cl, C2 and C3 at a dose of 8×10^2 conidia/insect (Table 2a,b). The lowest AST was 7.3 days for nymphs injected with conidia C2, followed by 8.4 days for C1 and 8.9 days for C3 (Table 2a,b). The *in vivo* passage of the isolate 90/2-Dm led to the most virulent conidia (C4), which had significantly lower AST than conidia C2, C1 and C3 and gave a value of 6.2 days (Table 2a,b). The mortality rates observed for conidia C1, C2, C3 and C4, were 46.25, 72.5, 27.5 and 80%, respectively (Figure 1).

The passage *in vitro* and *in vivo* of the strain 90/2-Dm also affected its toxicogenic activity. Table 3a,b shows the AST of controls and of locusts injected with 8 μl of crude filtrates Fl, F2, F3, and F4 at a dose of 500 μg mL^{-1} of total protein. Nymphs injected with the four types of filtrates showed AST significantly lower than the one of controls, whereas no significant differences were observed between the diverse crude filtrates (Table 3a,b). The highest and lowest values of mortality were 37.5% (F3) and 55% (F4), respectively (Figure 2).

Table 4a,b summarizes the AST of controls and locust injected with 8 μL of adialysates A1, A2, A3, and A4, at a concentration of 800 μg mL^{-1} of total protein. Injection of the four types of sources of macromolecules significantly reduced the AST of nymphs as compared to controls (Table 4a,b). Significant differences were also detected between the four types of adialysates. The lowest AST observed for nymphs injected with adialysate A4 was 5.5 days, followed by A2 (6.2 days), A3 (7.6 days), and A1 (8.3 days). Mortality rates observed for adialysates A1, A2, A3, and A4 were 37.5, 70, 55 and 72.5%, respectively (Figure 3). Dialysates D1, D2, D3, and D4 were not toxic to fourth instar nymphs of *L. migratoria*.

L. migratoria nymphs injected with crude filtrates or adialysates of isolate 90/2-Dm showed signs of weakness as early as 3 days after treatment, and they fed and developed at lower rates than the controls. Furthermore, most of the nymphs injected with the crude filtrates and adialysates remained at fourth instar, whereas most of the controls reached the fifth instar, although these data were not quantified. Observation of tissues of cadavers under binocular and light microscopes revealed melanized dark spots on tracheae, air sacs, and melanized nodules in the fat body in contact with the cuticle (Figure 4).

Insecticidal activity of the proteins from the crude filtrate of 90/2-Dm precipitated with ammonium sulphate

The AST of nymphs injected with 8 μL of the Sephadex G-25 fraction of 90/2-Dm at a dose of 1 mg mL^{-1} was 4.1 days, which was significantly lower (log rank = 306.94; $P < 0.0001$)

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TABLE 2. Susceptibility of fourth instar nymphs of *L. migratoria* to conidia of the strain 90/2-Dm of *B. bassiana* from a stock culture or collected after different passages. Nymphs inoculated by injection of 8×10^2 conidia per insect. Four replicates with 10 nymphs/replicate; whole experiment repeated two times

	Control	C1*	C2	C3	C4
(a) Kaplan–Meier analysis of average survival time (AST) in days					
AST (±SE)	9.53 (±0.18)	8.38 (±0.24)	7.30 (±0.22)	8.90 (±0.23)	6.20 (±0.22)
95% Conf. Int.	9.17, 9.88	7.91, 8.84	6.88, 7.72	8.46, 9.34	5.76, 6.63

(b) Log rank statistic and significance of survival times between treatments ($P \leq 0.05$ indicates significant difference)

	Control	C1	C2	C3
Cl	26.39 $P < 0.0001$			
C2	63.77 $P < 0.0001$	11.77 $P = 0.0006$		
C3	8.90 $P = 0.0029$	5.35 $P = 0.0208$	30.65 $P < 0.0001$	
C4	81.50 $P < 0.0001$	28.30 $P < 0.0001$	10.01 $P = 0.0016$	50.06 $P < 0.0001$

*The conidia designtated C1, C2, C3, and C4 were obtained from a stock slant culture, after two passages through MA, after two passages through SDA and after two passages through the host, respectively.

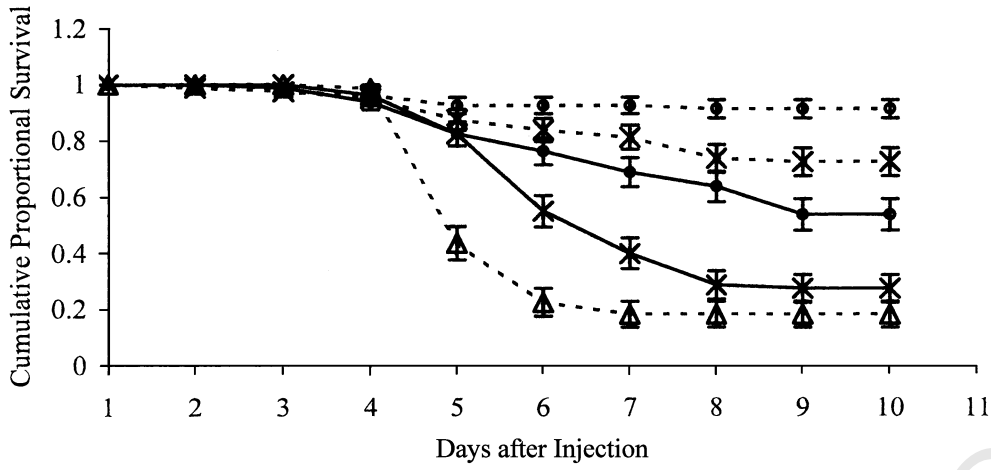

FIGURE 1. Cumulative proportional survival of fourth instar *L. migratoria* nymphs injected with conidia of strain 90/2-Dm of *B. bassiana* after passage through different culture media. Conidia C1 were obtained from a stock slant culture (solid line and ○), C2 after two passages through MA (solid line and ×), C3 after two passages through SDA (dashed line and ×), and C4 after two passages through the host (dashed line and Δ). In all treatments the dose was 8×10^2 conidia per insect. Control insects were injected with 8 μL of sterile distilled water (dashed line and ○). Data are mean ± SE.

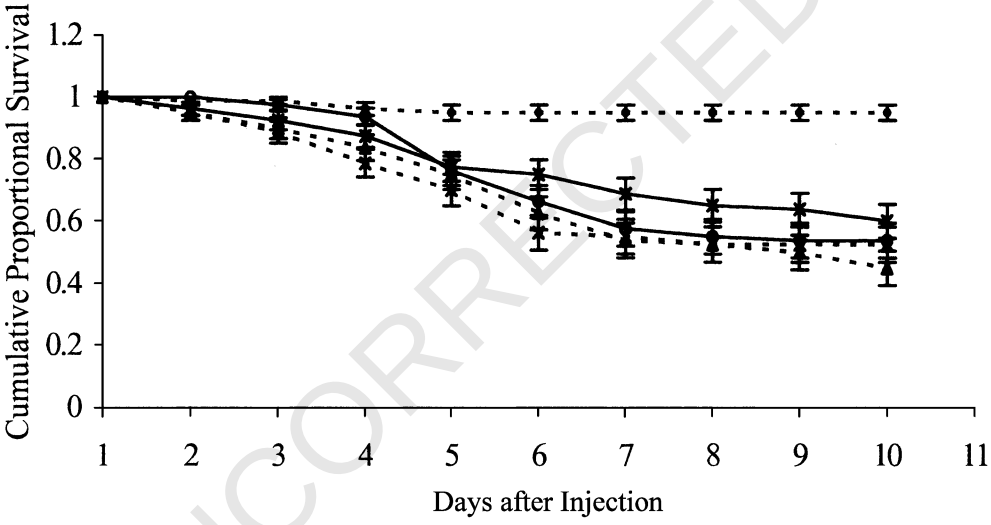

FIGURE 2. Cumulative proportional survival of fourth instar *L. migratoria* nymphs injected with 8 μL of different crude filtrates of strain 90/2-Dm. Crude filtrate F1 was obtained from conidia C1 (solid line and ○), F2 from conidia C2 (solid line and ×), F3 from conidia C3 (dashed line and ×), and F4 from conidia C4 (dashed line and Δ). Control insects were injected with 8 μL of sterile Adamek's liquid medium (dashed line and ○). The concentration of total proteins of the filtrates was 500 μg mL^{-1}. Data are mean ± SE.

than that of 9.6 days, observed for the controls. The Sephadex G-25 fraction was highly toxic causing a 98.7% mortality within l0 days insects injected with the G-25 desalted fraction

TABLE 3. Susceptibility of fourth instar nymphs of *L. migratoria* to different crude filtrates of cultures of isolate 90/2-Dm. Nymphs inoculated by injection of 8 µl of solution at a concentration of total protein of 500 µg mL^{-1}. Four replicates with 10 nymphs/replicate; whole experiment repeated two times

	Control	F1*	F2	F3	F4
(a) Kaplan–Meier analysis of average survival time (AST) in days					
AST (±SE)	9.69 (±0.15)	8.00 (±0.26)	8.26 (±0.29)	7.49 (±0.32)	7.63 (±0.31)
95% Conf. Int.	9.38, 9.99	7.49, 8.51	7.69, 8.84	6.86, 8.11	7.03, 8.22

(b) Log rank statistic and significance of survival times between treatments ($P \leq 0.05$ indicates significant difference)

	Control	F1	F2	F3
F1	34.10 $P < 0.0001$			
F2	27.26 $P < 0.0001$	0.51 $P = 0.4763$		
F3	36.49 $P < 0.0001$	0.39 $P = 0.5331$	1.41 $P = 0.2345$	
F4	46.44 $P < 0.0001$	1.25 $P = 0.2636$	3.40 $P = 0.0653$	0.27 $P = 0.6040$

*The Crude Filtrates designated F1, F2, F3, and F4 were obtained from cultures inoculated respectively with conidia C1, C2, C3, and C4 of the strain 90/2-Dm of *B. bassiana*.

TABLE 4. Susceptibility of fourth instar nymphs of *L. migratoria* to different adialysates of culturc of isolate 90/2-Dm of *B. bassiana*. Nymphs inoculated by injection of 8 μL at a concentration of total protein of 800 μg ml^{-1}. Four replicates with 10 nymphs/replicate; whole experiment repeated two times

	Control	Al*	A2	A3	A4
(a) Kaplan–Meier analysis of average survival time (AST) in days					
AST (±SE)	9.68 (±0.16)	8.29 (±0.27)	6.25 (±0.30)	7.63 (±0.30)	5.53 (±0.32)
95% Conf. Int.	9.36, 9.99	7.75, 8.83	5.66, 6.84	7.03, 8.22	4.90, 6.15

(b) Log rank statistic and significance of survival times between treatments ($P \leq 0.05$ indicates significant difference)

	Control	Al	A2	A3
A1	25.61 $P < 0.0001$			
A2	79.97 $P < 0.0001$	23.19 $P < 0.0001$		
A3	44.64 $P < 0.0001$	3.58 $P = 0.0586$	9.24 $P = 0.0024$	
A4	86.57 $P < 0.0001$	31.76 $P < 0.0001$	1.70 $P = 0.1927$	15.82 $P = 0.0001$

*The adialysates Al, A2, A3 and A4 were obtained by dialysis of crude filtrates Fl, F2, F3, and F4, respectively.

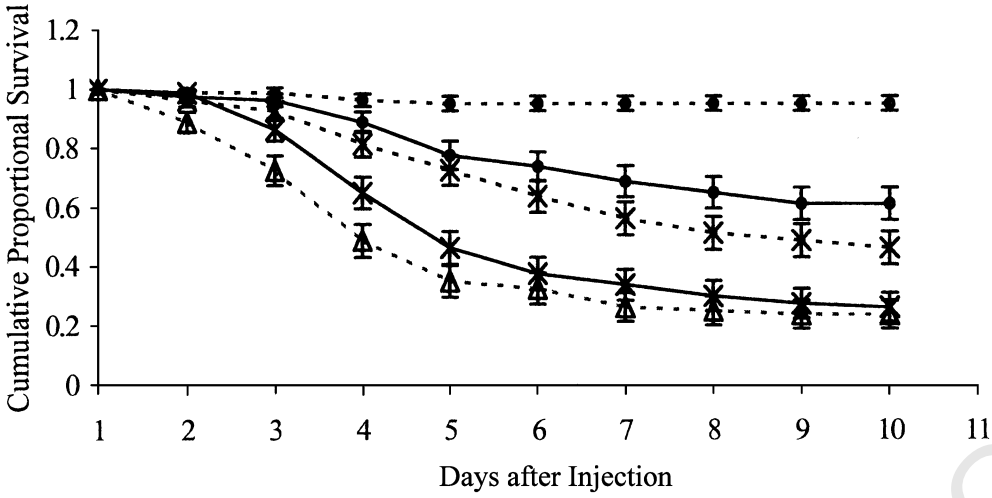

FIGURE 3. Cumulative proportional survival of fourth instar *L. migratoria* nymphs injected with 8μL of different adialysates of strain 90/2-Dm. Adialysate A1 was obtained from crude filtrate F1 (solid line and ○), A2 from crude filtrate F2 (solid line and ×), A3 from crude filtrate F3 (dashed line and ×), and A4 from crude filtrate C4 (dashed line and Δ). Control insects were injected with 8μL of sterile adialysate of Adamek's liquid medium (dashed line and ○). The concentration of total protein of the adialysates was 808 μg mL^{-1}. Data are mean ± SE.

showed the same symptoms when they were injected with crude filtrates and adialysates (Figure 4). In addition, a paralysis, characterised by an inability of the insect to right itself when placed on its dorsum was also observed.

The toxicity of the Sephadex G-25 fraction of the crude filtrate of isolate 90/2-Dm was significantly reduced by the two treatments at 60 and 120°C (Table 5). The untreated Sephadex G-25 traction had significantly lower AST (4.5 days) than when it was incubated 2 h at 60°C (7.7 days) and 20 min at 120°C (8.6 days), whereas no significant differences were observed between the two heat treatments. In addition, the Sephadex G-25 fraction caused over 95% mortality, which was significantly higher than the values of mortality observed for the same fraction after incubation 2 h at 60°C (50%) and 20 min at 120°C (35%)(Figure 5).

DISCUSSION

Research on toxic metabolites from our collection of isolates of *B. bassiana* is motivated by the importance of the analysis of their role in fungal pathogenesis. The selection of a fungal strain producing toxic metabolites that are implicated in its mode of action awaits the application of molecular techniques to improve its virulence and to develop an effective commercial mycoinsecticide (St Leger & Screen, 2001). An additional aim from an applied point of view, is the evaluation of the potential of these toxic fungal compounds as new bioinsecticides.

Isolates of *B. bassiana* varied considerably in their virulence and their ability to produce toxic metabolites *in vitro*. As our main objective was to examine the correlation between pathogenicity of a given isolate and its ability to produce toxic metabolites *in vitro*, all the bioassays were conducted by intrahaemocoelic injection, this method being the only valid one for such an approach (Roberts, 1981). This work shows that even under these experimental conditions, mortality rates can vary. Soil strains 0023-Su and 0026-Su were the most virulent, whereas strains isolated from *D. maroccanus* and *C. italicus* were moderately virulent. Surprisingly, isolates 902-Dm and 91/6-Ci are much more aggressive

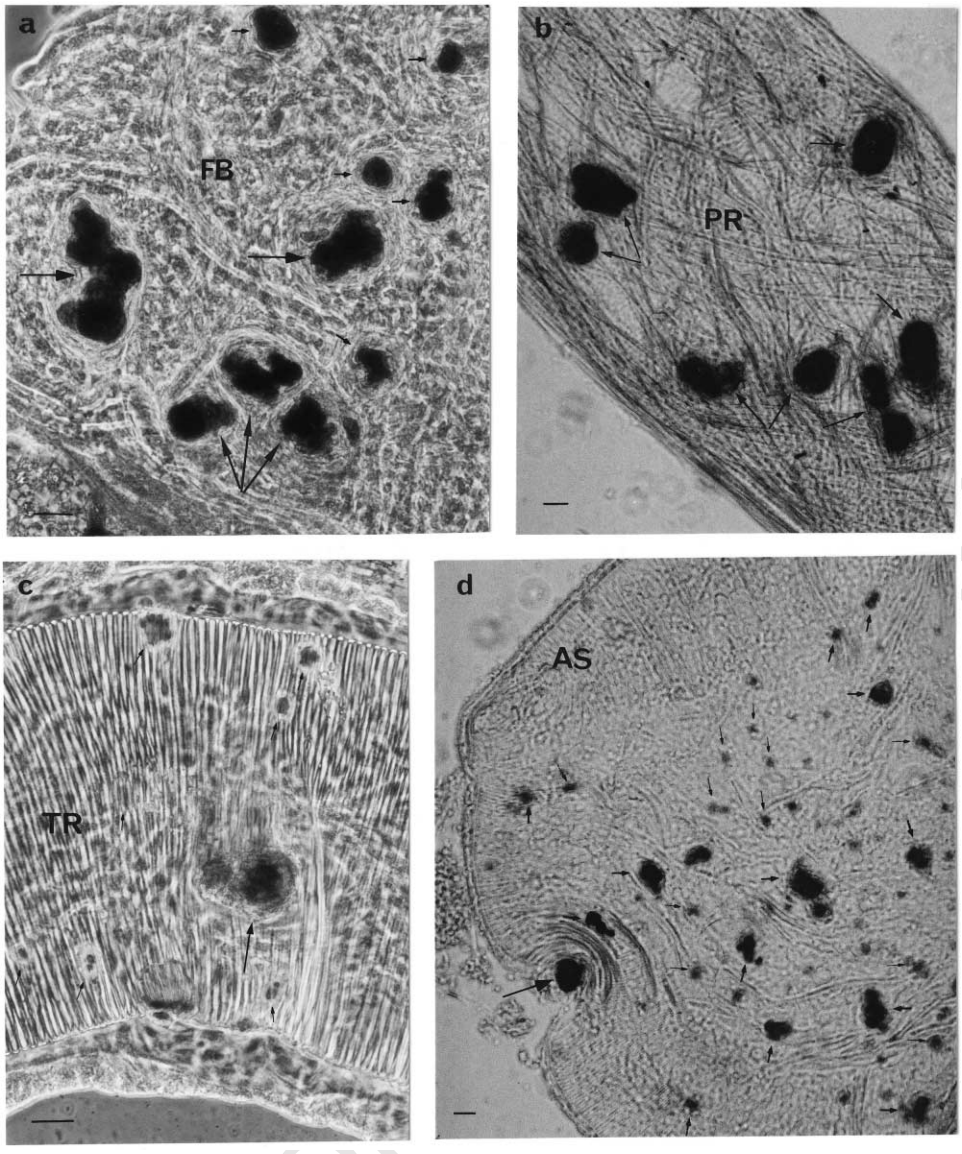

FIGURE 4. Effects of injection of different sources of toxic metabolites secreted *in vitro* by strain 90/2-Dm on different tissues of fourth instar nymphs of *L. migratoria*. (a) Melanized nodules (arrows) in the fat body (FB) induced by injection of 8 μL of crude filtrate F4 at a dose of 500 μg mL^{-1} 3 days post-treatment (bar:20 μm). (b) Melanized dark spots (arrows) on the proctodeum (PR) induced by injection of 8 μL of adialysate A4 at a dose of 800 μg mL^{-1} 4 days post-treatment (bar: 2 μm). (c) Melanized dark spots (arrows) on trachea (TR) induced by injection of 8 μL of adialysate A2 at a dose of 800 μg mL^{-1} 4 days post-treatment (bar: 20 μm). (d) Melanized dark spots (arrows) on an air sac (AS) induced by injection of 8 μL of the Sephadex G-25 desalted fraction at a dose of 1 mg mL^{-1} 4 days post-treatment (bar: 20 μm).

against *D. maroccanus* by contact exposure (Jiménez-Medina *et al*., 1998) than isolate 0023-Su (Santiago-Álvarez, unpublished data), which is only weakly pathogenic to the Moroccan locust, causing less than 20% mortality. Similar differences in virulence between contact and

TABLE 5. Effect of heat treatments on the insecticidal activity of the Sephadex G-25 fraction of strain 90/2-Dm against fourth instar nymphs of *L. migratoria*

	Control	G-25*	G-25 (2 h at 60°C)	G-25 (20 min at 120°C)
(a) Kaplan–Meier analysis of average survival time (AST) in days				
AST (±SE)	10 (±0.0)	4.50 (±0.36)	7.65 (±0.57)	8.55 (±0.33)
95% Conf. Int.	–	3.80, 5.20	6.54, 8.76	7.62, 9.48

(b) Log Rank statistic and significance of survival times between treatments ($P \leq 0.05$ indicates significant difference)

	Control	G-25	G-25 (2 h 60°C)
G-25	38.61 $P < 0.0001$		
G-25 (2 h 60°C)	13.17 $P = 0.0003$	15.40 $P < 0.0001$	
G-25 (20 min 120°C)	8.27 $P = 0.0040$	24.18 $P < 0.0001$	1.21 $P = 0.2718$[8]

*The concentration of total proteins of the Sephadex G-25 fraction of isolate 90/2-Dm was 1 mg mL^{-1}.

injection treatments have been also reported by Amiri-Besheli *et al*. (2000), suggesting the importance of the cuticle as a barrier to fungal infections.

Our results suggest that isolates of *B. bassiana* can be pathogenic for *L. migratoria* whether they have the ability to produce toxic metabolites *in vitro* or not. Among pathogenic isolates, only 90/2-Dm and 92/11-Dm secreted *in vitro* macromolecules toxic for *L. migratoria*, retained by dialysis with a membrane with a cut-off of 6–8 kDa for globular proteins. The crude filtrate of isolate 0023-Su was slightly toxic, whereas the adialysate was

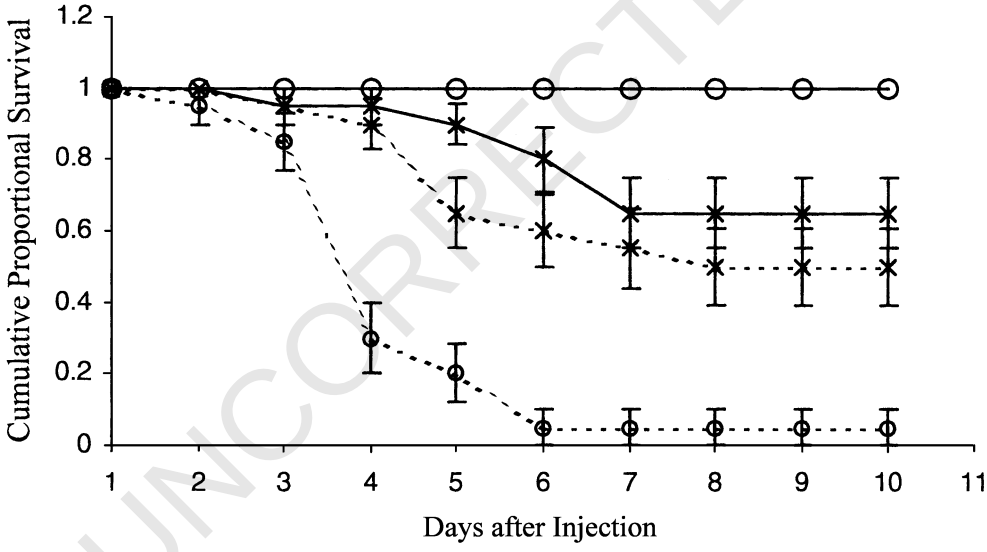

FIGURE 5. Cumulative proportional survival of fourth instar *L. migratoria* nymphs injected with 8μL of the Sephadex G-25 desalted fraction of strain 90/2-Dm of *B. bassiana* (dashed line and ○), with the same fraction incubated 2 h at 60°C (dashed line and ×) and 20 min at 120°C (solid line and ×). The total protein concentration was 1 mg mL^{-1} in the three treatments. 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 (solid line and ○). Data are mean±SE.

not. As the dialysate of this strain was the only one to show certain toxicity, an extraction of the crude filtrate with a conventional organic solvent used to extract small secondary metabolites would confirm if molecules such as cyclic peptides were responsible for this toxic activity.

Similarities can be noted between our data and the intra-specific variations already observed in the production of insecticidal cyclodepsipeptides of the group of destruxins by strains of *M. anisopliae* (Kershaw *et al*., 1999; Amiri-Besheli *et al*., 2000). Even if the majority of virulent strains of this fungal pathogen produce large quantities of destruxins, low mycotoxin producers may also be virulent, and exceptionally highly virulent strains may produce no destruxins *in vitro*. The strains that grow abundantly as hyphal elements in the haemolymph and do not cause destruxin-induced paralysis are considered by Kershaw *et al*. (1999) as representative of the 'growth strategy'. In contrast, the destruxin producing strains of *M. anisopliae*, as well as isolates 90/2-Dm, 92/11-Dm and 0023-Su of *B. bassiana*, could follow a 'toxin strategy'. However, the case of strains 90/2-Dm and 92/11-Dm is original, as the toxic metabolites involved are not small secondary metabolites but macromolecules. Further research is needed to check whether the macromolecular metabolites of strains 90/2-Dm and 92/11-Dm of *B. bassiana* are produced at physiologically active concentrations during their *in vivo* growth in *L. migratoria* and *D. maroccanus*. If the results of these investigations would be positive, these molecules could be considered as true 'vivotoxins' (Roberts, 1981).

Strain 90/2-Dm was chosen for further toxicological studies because it had also demonstrated a strong insecticidal activity on *D. maroccanus* by *per os* or contact exposure (Hernández-Crespo & Santiago-Álvarez, 1997; Jiménez-Medina *et al*., 1998). Our investigations have demonstrated that the toxic activity detected in culture filtrates of isolate 90/2-Dm was not due to low molecular mass metabolites such as cyclopeptides, organic acids or pigments which are known to be produced by *B. bassiana* and other fungi (Vey *et al*., 2001). The insecticidal activity was exclusively detected in high molecular weight fractions after dialysis or gel filtration on Sephadex G-25. Furthermore, the precipitation of the toxic metabolites in the crude filtrate of strain 90/2-Dm by 90% saturation of ammonium sulphate, and the reduction of their toxicity by the two thermal treatments, strongly support the assumption that these metabolites were proteins.

Further investigations are presently carried out to try to purify and characterize the macromolecular toxins secreted *in vitro* by 90/2-Dm. Preliminary results indicate that the biochemical properties of partially purified toxic fractions are different from those of Hirsutellin A, the only high molecular weight compound that has so far been purified to homogeneity (Mazet & Vey, 1995), and from other proteic macromolecules secreted by strains of *B. bassiana* active against the order Lepidoptera (Vey, unpublished data). The complete purification and characterisation of these active macromolecules will open the opportunity to characterise the cytotoxic effects already detected on cells in living locusts as well as in invertebrate cell cultures, without the interference of other molecules present in the crude sources. Furthermore, by using a specific antiserum, it should be possible to determine if these metabolites are produced *in vivo* and to establish if they have any role in pathogenesis. It also will allow to extend the studies to other strains and to determine if there is a common feature in the profile of metabolites specifically secreted against locusts by different strains of *B. bassiana***.** Moreover, investigations at the level of the genes encoding for these toxic compounds, as already performed for other virulence factors, namely the PR1 protease of *M. anisopliae* **(**St Leger *et al*., 1992), are a very promising perspective.

Previous studies conducted with the aim to develop a mycoinsecticide for the control of *D. maroccanus* (Hernández-Crespo & Santiago-Álvarez, 1997; Jiménez-Medina *et al*., 1998) have focused on the optimisation of the type and quantity of propagules, with practically no consideration given to the quality of inocula. The data reported here provide novel

information regarding the effect of subculturing on the quality of the conidia produced by this fungus.

Our results demonstrate that passage of *B. bassiana* 90/2-Dm strain through mycological media and through the host affected its virulence and the toxicity of the macromolecules secreted in its cultures. Attenuation or enhancement of virulence of *B. bassiana* following repeated subculturing in artificial media (Kawakami, 1960; Schaerffenberg, 1964), and maintenance of virulence (Ferron *et al.* 1972; Fargues & Robert, 1983; Brownbridge *et al*., 2001) have been previously reported. From these studies, it appears that the effect of repeated *in vitro* subculture of entomopathogenic fungi on their virulence varies depending on species and strains. Our work shows that the type of mycological media has also an influence on the consequences of *in vitro* subculturing; thus, two passages through SDA caused a decline in virulence of conidia while two passages through MA resulted in conidia more virulent than those from the stock slant cultures. The differences in nutritional composition between SDA and MA might explain the difference in the observed response. It is generally accepted that there is a reduction in the capacity of a fungal isolate to sporulate in nutrient-rich media like SDA (Schaerffenberg, 1964), probably due to an adaptation of the fungus to its saprophytic phase, resulting in a progressive attenuation of its virulence. Strain 90/2-Dm shows the highest production of mycelium in SDA (Quesada-Moraga & Vey, unpublished data); thus such an adaptation probably occurred during our assays.

The number of passages is a factor that may have an impact on the effect of this kind of treatment. Interestingly, we have observed a significant increase in virulence of strain 90/2-Dm after only two passages through MA. Similarly, Schaerffenberg (1964) observed that the maximum virulence of a strain of *B. bassiana* was reached at the third generation on nutrient medium, it was maintained at the same level up to the 16th generation, and from that point, virulence declined until the 30th generation. As our number of passages was low, further quantitative studies would be useful to document effects of additional *in vitro* passages beyond two for 90/2-Dm, and other strains of *B. bassiana*.

B. bassiana is a facultative and opportunistic parasite. Therefore, during culture, the parasitic phase in the life cycle of this entomopathogenic fungus cannot be left out without risk of loss of virulence. For this reason, it is recommended to interrupt artificial culture from time to time by passage through insect hosts (Müller-Kögler, 1966; Aizawa, 1971). Increased virulence after a few passages through insects has also been reported for other entomopathogens (Kawakami, 1960; Hartmann & Wasti, 1974; Wasti & Hartmann, 1975; Fargues & Robert, 1983). However, conflicting results have also been obtained by other authors, as Latch (1976) and Morrow *et al*. (1989), who did not observe enhancement of virulence of *B. bassiana* after passage through insect hosts.

Our data are in agreement with others reporting a positive effect of passage through the host on virulence, as in our experimental conditions strain 90/2-Dm exhibited the highest level of virulence after two passages through *L. migratoria*. As this strain was originally isolated from *D. maroccanus*, such results suggest that passage through *L. migratoria* might also have led to an adaptation to this alternative host.

The passages through mycological media and through the host also affected the toxicity of the metabolites produced *in vitro* by isolate 90/2-Dm of *B. bassiana.* This is the first study to establish the effect of the *in vitro* subculturing of entomogenous fungi on this metabolic process. No significant differences in toxicity were observed among the four types of crude filtrates containing all types of fungal molecules, whereas the insecticidal activity of adialysates was clearly determined by the type of conidia used for the inoculation of cultures. The differences in toxicity between crude filtrates and adialysates cannot be attributed to differences in total protein concentration as similar quantities were injected. The disparity between the two types of sources of fungal metabolites probably reflects differences in their chemical composition. As adialysates represent a first level of increased purity and as risk of interference between macromolecules and low molecular weight

components of the filtrate had disappeared as a consequence of dialysis, the bioassays performed with adialysates were more useful to reveal the changes which may occur in the biosynthesis of macromolecular toxic metabolites.

Our results indicate that the *B. bassiana* 90/2-Dm strain is stable enough to be submitted to a limited number of repeated transfers on MA, as could occur during the mass-production process, without any adverse effects on its virulence and ability to produce toxic macromolecular metabolites. This stability is another favourable characteristic of this strain, in addition to its virulence and toxicogenic activity; thus we consider the 90/2-Dm strain of *B. bassiana* as a good candidate for improving its performance and for developing a marketable agent for the control of *D. maroccanus*.

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