Selection of *Beauveria bassiana* and *Metarhizium anisopliae* Isolates to Control *Triatoma infestans*

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Twenty three isolates of Beauveria bassiana *and 13 isolates of* Metarhizium anisopliae *were tested on third instar nymphs of* Triatoma infestans, *a serious vector of Chagas disease. Pathogenicity tests at saturated humidity showed that this insect is very susceptible to fungal infection. At lower relative humidity (50%), conditions expected in the vector microhabitat, virulence was significantly different among isolates. Cumulative mortality 15 days after treatment varied from 17.5 to 97.5%, and estimates of 50% survival time varied from 6 to 11 days. Maintaining lower relative humidity, four* B. bassiana *and two* M. anisopliae *isolates were selected for analysis of virulence at different conidial concentrations and temperatures. Lethal concentrations sufficient to kill 50% of insects (LC_{50}) varied from $7.1x10^5$ to $4.3x10^6$ conidia/ml, for a* B. bassiana *isolate (CG 14) and a* M. anisopliae *isolate (CG 491) respectively. Most isolates, particularly* B. bassiana *isolates CG 24 and CG 306, proved to be more virulent at 25 and 30°C, compared to 15 and 20°C. The differential virulence at 50% humidity observed among some* B. bassiana *isolates was not correlated to phenetic groups in cluster analysis of RAPD markers. In fact, the* B. bassiana *isolates analyzed presented a high homogeneity (> 73% similarity).*

Key words: vector control - Chagas disease - entomogenous fungi - virulence - random amplification of polymorfic DNA

Entomopathogenic fungi are promising candidates for microbiological control of Triatominae (Hemiptera, Reduviidae) because they invade their hosts through the integument. However, relative humidity (RH) and temperature are known to be limiting environmental factors for fungal development on insects (Glare & Milner 1991, Ferron et al. 1991). High rates of infection and a rapid kill of triatomine bugs by the hyphomycete fungi *Beauveria bassiana* and *Metarhizium anisopliae* were obtained at humidities close to saturation (Silva & Messias 1985, Romaña & Fargues 1987, Luz 1990, Romaña 1992, Luz et al. 1994). Infection of bugs diminished with *B. bassiana* at RH below 97% (Luz 1994). Optimal temperatures for fungal development on the insect host range from 16 to 30°C for *B. bassiana* and *M. anisopliae* with a faster development at the higher temperatures (Ferron et al. 1991).

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Microclimatic conditions in the natural insect habitat may be unfavorable for fungal infection. Humidity in domestic microhabitats of triatomine bugs may be distinctly lower as shown by Luz (1994) in *Rhodnius prolixus*. To select isolates for biocontrol, intraspecific differences of fungal behaviour related to abiotic conditions in target insect habitats should be considered. In this study, isolates of *B. bassiana* and *M. anisopliae* were screened against *Triatoma infestans* at RH close to saturation and at 50% RH. The effect of conidial concentrations and temperatures were analyzed for some isolates at the lower RH. Polymorphism among some *B. bassiana* isolates, with different levels of virulence, was investigated by using random amplification of polymorphic DNA (RAPD) analysis.

MATERIALS AND METHODS

Insect rearing - *T. infestans* was mass-reared in the laboratory. Insects were allowed to feed on chickens every two weeks, and maintained at 25±0.5°C, 75±5% RH with a photophase of 12 hr. The *T. infestans* colony was originally from the State of Paraná, Brazil, and has been maintained in laboratory since 1981.

Fungal cultures - Most of the 23 *B. bassiana* and 13 *M. anisopliae* isolates selected for this study

were originally obtained from hemipteran insects in Brazil (Table I). Conidia were obtained from cultures grown on complete media (0.001 g $FeSO_4$, 0.5 g KCl, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$.7 H_2O, 6 g $NaNO_3$, 0.001 g $ZnSO_4$, 1.5 g hydrolysed caseine, 0.5 g yeast extract, 10 g glucose, 2 g peptone, 20 g agar and 1 l distilled water) at 27°C for 15 days. Mycelium used for RAPD analysis was obtained from a submersed culture of conidia in complete medium shaking at 150 rpm at 25°C for three days. Mycelium was harvested by filtration through filter paper (Whatman No. 1), lyophilized and stored at -80°C.

Bioassays - Conidia were harvested from plate cultures and suspended in sterile distilled water with 0.1% Tween 80 (Sigma, St. Louis, MO, USA). Newly emerged and unfed third instar *T. infestans* nymphs were used in the assays. Tests on pathogenicity at a RH close to saturation and 25°C were done by immersion of ten insects in a conidial suspension (10^8 conidia/ml) for approximately 6 sec. For all other assays the same method of application was used with four replicates of ten insects. A suspension of 10^7 conidia/ml was used to evaluate virulence of isolates at 50% RH and 25°C. Maintaining this lower RH, six selected isolates (CG 14, CG 24, CG 144, CG 306, CG 474 and CG 491) were tested at four temperatures (15, 20, 25 and 30°C) and seven conidial concentrations (10^5, $3x10^5$, 10^6, $3x10^6$, 10^7, $3x10^7$ and 10^8 conidia/ml). Control insects were treated as described above but without conidia. After treatment, insects were transferred to gauze-covered transparent cups (55 mm diameter x 75 mm) and kept in a chamber with regulated temperature and humidity (50±5% RH and 25±1°C). For tests at different temperatures, insects were held in desiccators (53% RH), which were kept in incubators. Humidity inside desiccators was maintained by using a saturated aqueous solution of $MgNO_3.6H_2O$ (Winston & Bates 1960). For all assays, mortality of insects was recorded daily during 15 days after treatment.

RAPD analysis - Genomic DNA of ten *B. bassiana* isolates (CG 14, CG 16, CG 19, CG 21, CG 24, CG 136, CG 261, CG 306, CG 474 and CG 516) was obtained using a universal rapid salt extraction method (Aljanabi & Martinez 1997). PCR reactions were performed in 50-ml volumes, with 15 ng of each template, using the PTC-100 programmable thermal controller (MJ Research), and a temperature profile described by Tigano-Milani et al. (1995). Amplifications were done using the following reaction mix: 2 units of Taq polymerase (Cenbiotec), 5 ml of 10x Taq polymerase reaction buffer, 200 mM of each deoxynucleotides triphosphate (Pharmacia Biotec), and 0.4 mM of 10-mer primer (Operon Technologies, Alameda CA.). Ten primers were selected for the analysis: OPE-01, OPE-02, OPE- 03, OPE-04, OPE-07, OPE-14, OPE-15, OPE-16, OPE-19 and OPE-20. Amplified products were separated by electroforesis in 2% LE agarose gel dissolved in 0.5x Tris-borate-EDTA (TBE) buffer. After electrophoresis, gels were stained with ethidium bromide (Sambrook et al. 1989) and photographed under UV light. DNA fingerprints were scored directly from the photographs.

Data analysis - Angular transformed cumulative mortalities were analyzed by ANOVA (analysis of variance) and means compared by cluster analysis (Scott & Knott 1974). Estimates of 50% survival time were calculated (Lee 1980), curves of survival analyzed by log-rank-test and compared by Z-statistics (Fox 1993). Lethal concentrations to kill 50% and 90% (LC_{50} and LC_{90}) were calculated by probit analysis (SAS Institute Inc. 1989). RAPD characters were analyzed using NTSYS-pc V1.8. A similarity matrix was created using the Jaccard similarity coefficient (Sneath & Sokal 1973). Clustering was done using the unweighted mean pair group arithmetic mean method (UPGMA).

RESULTS

Effect of humidity on mortality - Most *B. bassiana* and *M. anisopliae* isolates tested at RH nearing saturation and 25°C, induced cumulative mortalities between 90 and 100% in third instar nymphs of *T. infestans* (Table II). At 50% RH, the virulence of most isolates was reduced, but four *B. bassiana* isolates (CG 21, CG 306, CG 474 and CG 516) and two isolates of *M. anisopliae* (CG 144 and CG 491) caused mortalities of 90% or higher. Four other *B. bassiana* isolates (CG 14, CG 19, CG 24 and CG 261) and one isolate of *M. anisopliae* (CG 50) caused mortalities over 85% at 50% RH. ANOVA of mortalities showed a significant difference between *B. bassiana* isolates ($F = 4.8$, $p < 0.0001$), but not between isolates of *M. anisopliae* ($F = 1.4$, $p = 0.1928$). Comparison of means resulted in three groups ($\alpha = 0.05$) for *B. bassiana* isolates. Estimates of 50% survival time of insects varied from six days, for CG 550, to eleven days for CG 19, CG 42 and CG 125. A significant difference between survival curves of *B. bassiana* isolates ($\chi^2 = 42.9$, $p < 0.0001$) and *M. anisopliae* isolates ($\chi^2 = 37.9$, $p < 0.0001$) was detected.

Effect of temperature and conidial concentration on mortality - The effect of temperature and conidial concentration on fungal virulence were analyzed at 50% RH. Four isolates of *B. bassiana* (CG 14, CG 24, CG 306, CG 474) and two isolates of *M. anisopliae* (CG 144, CG 491) were selected

TABLE I

Host origin and geographical location of *Beauveria bassiana* and *Metarhizium anisopliae* isolates

Species/Isolate [a]	Geographical location	Host or substrate city/country	Year
B. bassiana			
CG 06	Pelotas/Brazil	Soil	1991
CG 14	Londrina/Brazil	*Podisus* sp. [b]	1988
CG 16	Ipojuca/Brazil	*Diatraea saccharalis* [c]	1983
CG 19	Londrina/Brazil	*Nezara viridula* [b]	1981
CG 21	France	Unidentified Pentatomid [b]	1973
CG 22	Londrina/Brazil	*Nezara viridula* [b]	1983
CG 24	Londrina/Brazil	*Euschistus heros* [b]	1986
CG 25	Brasília/Brazil	*Anticarsia gemmatalis* [c]	1987
CG 78	Yerba Buena/Argentina	*Nezara viridula* [b]	1986
CG 135	Brazil	*Euselasia* sp. [c]	1988
CG 136	Piracicaba/Brazil	Unidentified Cercopidae [b]	NA
CG 149	Goiânia/Brazil	*Deois flavopicta* [b]	1982
CG 154	Jataí/Brazil	*Deois flavopicta* [b]	1983
CG 261	Brasília/Brazil	*Edessa meditabunda* [b]	1992
CG 287	Brasília/Brazil	*Nezara viridula* [b]	1992
CG 306	Brasília/Brazil	*Thyanta perditor* [b]	1990
CG 470	Cascavel/Brazil	*Podisus* sp. [b]	1984
CG 474	Londrina/Brazil	*Podisus* sp. [b]	1984
CG 479	Santana do Ipanema/Brazil	Unidentified Vespidae [d]	1985
CG 516 (Bb 66) [f]	Oliveros/Argentina	*Nezara viridula* [b]	1992
CG 517 (Bb 67) [f]	Oliveros/Argentina	*Nezara viridula* [b]	1992
CG 549 (Bb 252) [g]	Italy	*Adelphocoris* sp. [b]	1983
CG 550 (Bb 297) [g]	Poland	Unidentified Heteroptera [b]	1971
M. anisopliae			
CG 40	Brasília/Brazil	*Deois flavopicta* [b]	1988
CG 41	Brasília/Brazil	*Nezara viridula* [b]	1987
CG 42	Brasília/Brazil	*Deois flavopicta* [b]	1989
CG 46	Brazil	*Deois incompleta* [b]	NA
CG 50	Brasília/Brazil	*Deois flavopicta* [b]	1987
CG 93	Brasília/Brazil	*Deois flavopicta* [b]	1985
CG 97	Brasília/Brazil	Unidentified Scarabaeidae [e]	1988
CG 125	Santa Izabel/Brazil	*Monalonion annulipes* [b]	1991
CG 144	Goiânia/Brazil	*Piezodorus guildinii* [b]	1982
CG 167	Goiânia/Brazil	*Tibraca limbativentris* [b]	1985
CG 339	Brasília/Brazil	Unidentified Scarabaeidae [e]	1991
CG 491	Londrina/Brazil	*Deois* sp. [b]	1983
CG 498	Goiatuba/Brazil	*Scaptores castanea* [b]	1983

a: Embrapa/Cenargen Collection, Brasília, Brazil; *b*: Hemiptera; *c*: Lepidoptera; *d*: Hymenoptera; *e*: Coleoptera, NA: not available; *f:* isolates obtained from INTA/IMYZA Collection, Castelar, Argentina; *g*: isolates obtained from INRA Collection, Montpellier, France.

for this study. Progress of mortality at different temperatures is demonstrated in Fig. 1. There was a significant effect of the isolate ($F = 15.7$, $p < 0.0001$) and temperature ($F = 14.8$, $p < 0.0001$) on insect cumulative mortality, 15 days after treatment. Within all temperatures tested, mortality due to the *B. bassiana* isolates was higher than in the *M. anisopliae* isolates (7.2 %, I.C. 95 % at 3.9-11.3 %). Isolates showed different patterns of mortality at increasing temperatures ($F = 2.6$, $p < 0.004$) with a significant linear effect in CG 14, CG 144 and CG 491, a significant linear and quadratic effect in CG 24 and CG 306. No significant differences between mortalities at different temperatures were observed for CG 474. The values of LC_{50}, 15 days after fungal application, varied from $7.1x10^5$ to $4.3x10^6$ conidia/ml for a *B. bassiana* isolate (CG 14) and a *M. anisopliae* isolate (CG 491) respectively (Table III). Values of LC_{90} varied between $4.6x10^6$ (CG 474) and $1.4x10^8$ (CG 491). Confi-

TABLE II

Virulence of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to *Triatoma infestans at* 25°C and different relative humidities (RH)

Species/Isolate	RH close to saturation Mortality (%)[a]	50% RH[b]	
		Mortality (%)[c]	Estimates of 50% survival time (days)[d]
B. bassiana			
CG 21	100	97.5 (2.5)[a]	7.0 (7; 8)[b]
CG 306	100	97.5 (2.5)[a]	8.0 (7; 9)[ab]
CG 516	100	97.5 (2.5)[a]	9.0 (7; 10)[ab]
CG 474	100	92.5 (7.5)[a]	8.0 (7; 9)[ab]
CG 19	100	87.5 (6.2)[a]	11.0 (10; 11)[a]
CG 14	100	85.0 (8.7)[a]	9.0 (8; 11)[b]
CG 24	100	85.0 (15.0)[a]	8.0 (8; 10)[b]
CG 261	90	85.0 (8.7)[a]	8.5 (7; 9)[b]
CG 149	100	82.5 (17.5)[a]	10.0 (9; 10)[a]
CG 22	100	80.0 (14.1)[a]	7.0 (6; 7)[b]
CG 78	100	80.0 (16.8)[a]	8.0 (7; 10)[b]
CG 06	100	72.5 (11.1)[a]	9.5 (8; 11)[a]
CG 550	100	67.5 (4.8)[a]	6.0 (6; 8)[ab]
CG 470	100	65.0 (11.9)[a]	10.0 (9; 12)[a]
CG 517	100	62.5 (14.9)[b]	*
CG 549	100	57.5 (11.1)[b]	*
CG 154	100	55.0 (19.4)[b]	*
CG 25	100	52.5 (10.3)[b]	*
CG 16	50	45.0 (20.2)[b]	*
CG 136	100	45.0 (12.6)[b]	*
CG 287	100	35.0 (8.7)[c]	*
CG 135	100	25.0 (6.5)[c]	*
CG 479	100	17.5 (2.9)[c]	*
M. anisopliae			
CG 144	100	90.0 (4.1)	7.0 (6; 7)[b]
CG 491	100	90.0 (10.0)	7.0 (7; 9)[ab]
CG 50	100	85.3 (5.0)	9.5 (8, 10)[ab]
CG 93	100	82.5 (7.5)	10.0 (8; 11)[ab]
CG 125	100	80.0 (13.5)	11.0 (10; 13)[a]
CG 41	100	77.5 (13.2)	9.5 (8; 11)[ab]
CG 42	90	77.5 (6.3)	11.0 (10; 11)[a]
CG 97	90	67.5 (6.3)	9.0 (8; 11)[a]
CG 40	100	65.0 (11.9)	10.0 (8; 12)[a]
CG 339	90	65.0 (15.0)	9.5 (8; 15)[a]
CG 167	90	60.0 (14.7)	*
CG 46	90	57.5 (16.0)	8.0 (7; 9)[ab]
CG 498	90	45.0 (10.4)	*

a: cumulative % mortality, 15 days after treatment of 10 third instar nymphs treated with 10^8 conidia/ml of each isolate; *b*: tests were done with four replicates of 10 third instar nymphs/replicate, using 10^7 conidia/ml of each isolate; *c*: cumulative % mortality, 15 days after treatment (standard error of observed mean). Means, analyzed in arc sin scale, followed by the same letter were not significantly different (cluster analysis method, designed by Scott & Knott 1974); *d*: estimates of 50% survival time (days) (C.I. 95%). Values, followed by the same letter, did not differ significantly in survival curves at an overall 0.1 significance level. *: mortality rate was not enough to estimate 50% survival time.

dence intervals indicate similarity in virulence among *B. bassiana* isolates, but not in *M. anisopliae* isolates.

RAPD analysis - The ten primers used for the *B. bassiana* isolates produced 114 scorable bands, and the average genetic similarity among these isolates was 79%. Cluster analysis of the RAPD data did not produce well defined phenetic groups (Fig. 2). The isolates analyzed presented high similarity (> 73%), although they had been selected for presenting different virulence towards *T. infestans* (Table II).

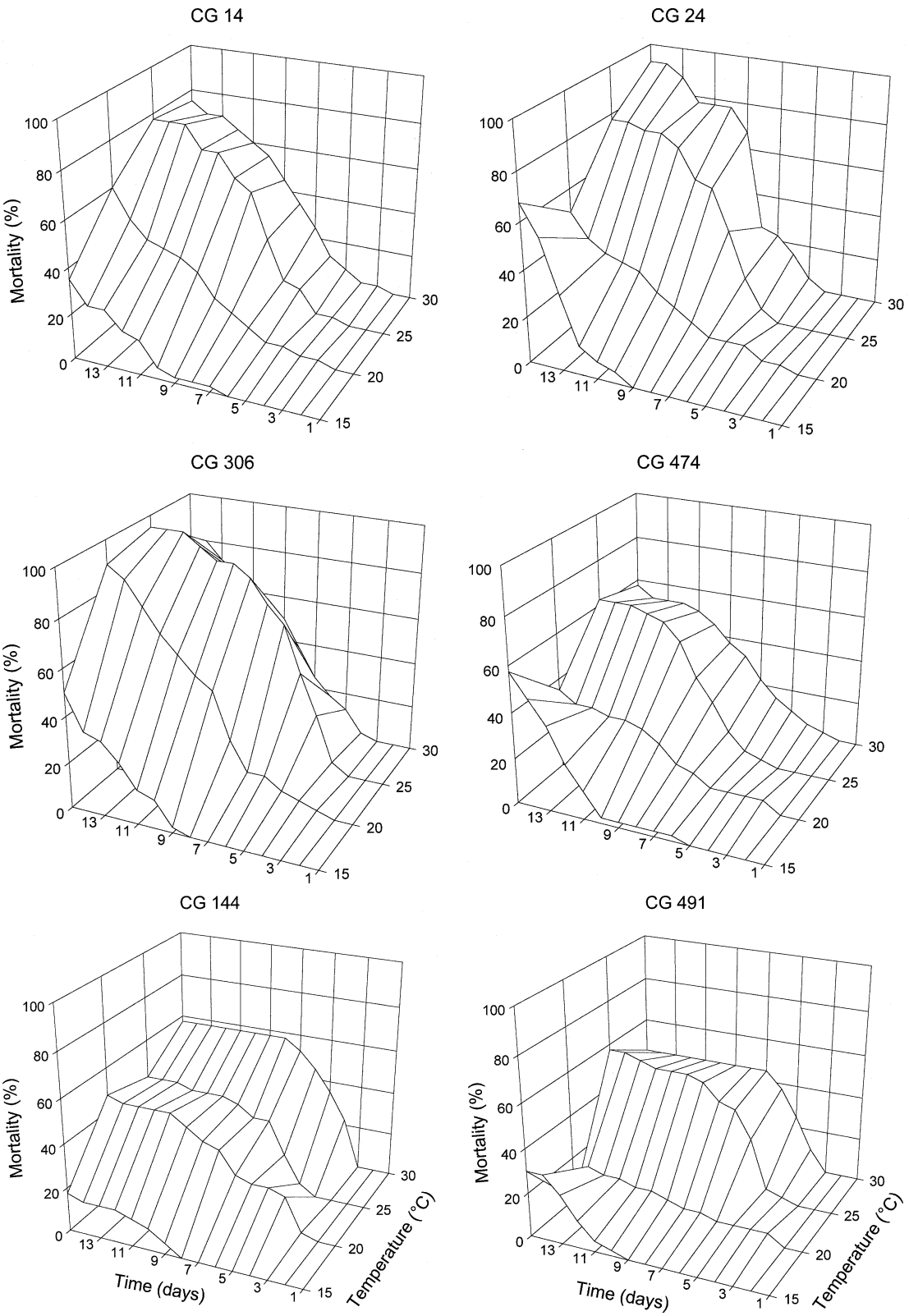

Fig. 1: cumulative mortality of *Triatoma infestans* third instars treated with *Beauveria bassiana* (CG 14, CG 24, CG 306 and CG 474) and *Metarhizium anisopliae* (CG 144 and CG 491) isolates at 15, 20, 25 and 30°C and 53% relative humidity.

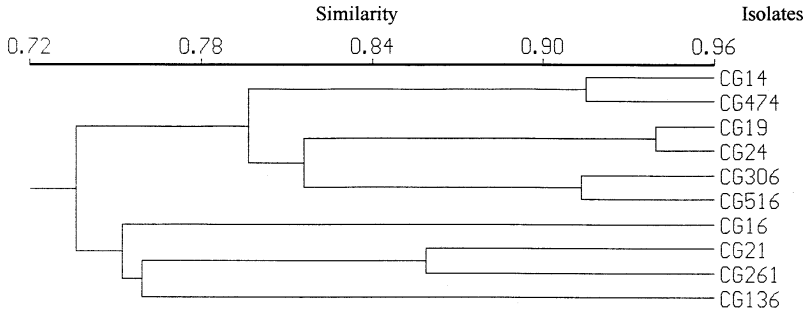

Fig. 2: dendrogram constructed from RAPD data, indicating the relationships among *Beauveria bassiana* isolates. A similarity matrix was calculated using the Jaccard coefficient, and the tree was generated from this matrix by unweighted pair group method, arithmetic mean (UPGMA).

TABLE III

Lethal concentrations of *Beauveria bassiana* and *Metarhizium anisopliae* isolates for *Triatoma infestans*

Species/Isolate	LC_{50} (C.I. 95%)	LC_{90} (C.I. 95%)
B. bassiana		
CG 14	0.7 (0.3-1.4)	6.0 (2.9-18.0)
CG 24	1.3 (0.6-2.3)	8.3 (4.5-36.0)
CG 306	0.9 (0.6-1.2)	7.2 (5.6-11.0)
CG 474	1.0 (0.3-2.2)	4.6 (2.5-13.0)
M. anisopliae		
CG 144	1.8 (1.4-2.4)	10.0 (7.2-16.0)
CG 491	4.3 (2.7-6.8)	140 (68.0-400.0)

LC_{50} and LC_{90} (C.I.) based on seven concentrations of conidia (10^5, $3x10^5$, 10^6, $3x10^6$, 10^7, $3x10^7$ and 10^8 conidia/ml) were calculated 15 days after treatment. All values multiplied by 10^6. Tests were done with four replicates of 10 third instar nymphs each, at 25°C and 53% relative humidity.

DISCUSSION

All *B. bassiana* and *M. anisopliae* isolates tested proved to be pathogenic to *T. infestans* at a RH nearing saturation. However, several isolates of *B. bassiana* and *M. anisopliae* were also virulent against *T. infestans* at 50% RH. High mortalities due to infection with *B. bassiana* independent of RH or at low RH was reported for other insect pests (Ferron 1977, Doberski 1981, Marcandier & Khachatourians 1987) and has also been observed for other fungal species (Hsiao et al. 1992, Fargues et al. 1997). Exposure of fungus-treated triatomine bugs to undefined humidities resulted in low rates of insect mortality (Dias & Leão 1967, Romaña & Romaña 1981, Sherlock & Guitton 1982). Only Luz (1990) and Romaña (1992) reported a somewhat superior susceptibility of *R. prolixus* to *B. bassiana* at 40% RH, compared to higher humidities. Two *B. bassiana* isolates, CG 449 and CG 550, which were reported to be highly virulent to *T. infestans* and *R. prolixus* respectively at RH close to saturation (Romaña & Fargues 1987, Romaña 1992), were distinctly less virulent in the present study when tested at 50% RH and 25°C.

The four *B. bassiana* isolates, CG 14, CG 24, CG 306 and CG 474 and *M. anisopliae* isolate CG 144 showed no difference in effectiveness against *T. infestans* according to concentration of conidia applied. Only *M. anisopliae* isolate CG 491 was distinctly less virulent. Effectiveness of most isolates tested, particularly of the *M. anisopliae* isolates (CG 144 and CG 491), was reduced at 50% RH and temperatures of 15 or 20°C. The most virulent isolate at temperatures of 20 and 25°C, temperatures found in domestic habitats of *T. infestans*, and 50% RH was CG 306, which showed also an elevated virulence at 15 and 30°C. However, isolates CG 14 and CG 24 were the most active at 25 and 30°C. Virulence of the isolate CG 474 to *T. infestans* proved to be independent of the temperatures tested but was generally reduced compared to the other *B. bassiana* isolates.

Mietkiewski et al. (1994) found maximal mortality in *Galleria melonella* treated with *M. anisopliae* at 30°C. However, the two *M. anisopliae* isolates tested against *T. infestans* were not more virulent at 30°C compared to lower temperatures. The intraspecific optimum of temperature for fungal development can vary notably as shown by Moorhouse et al. (1994) who reported a *M. anisopliae* isolate with highest virulence against the vine weevil, *Otiorhynchus sulcatus*, at 10°C and another isolate with an optimum at 25°C. Recently Vidal et al. (1997) showed the correlation of geographic origin of *Paecilomyces fumosoroseus* isolates and their temperature ranges in terms of vegetative growth on artificial media.

RAPD analysis indicated that the *B. bassiana* isolates were quite homogeneous, despite their differences in virulence against *T. infestans*. The virulent isolates were even more homogenous and could not be distinguished by these molecular markers. The analysis of isoenzymes of *B. bassiana* isolates has shown that it is not possible to correlate molecular polymorphism with virulence (Lecuona et al. 1996) This high similarity could be related to the original host of the strains analyzed. With the exception of CG 16, all others were isolated from heteropteran insects. Maurer et al. (1997) have shown, by RFLP and RAPD analysis, clear relationships between the population structure of *B. bassiana* and some defined host species. However, a larger sample number of isolates from different origins should be used to allow the identification of related groups.

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