COMUNICAÇÃO CIENTÍFICA

CHARACTERIZATION AND VIRULENCE OF *Metarhizium anisopliae* (METSCH.) SOROK. ISOLATES TO EGGS OF *Deois flavopicta* (STAL) (HOMOPTERA: CERCOPIDAE)

Marcos R. de Faria[1], Myrian S. Tigano[1], Eliana M.G. Fontes[1] e Roberto E. Lecuona[2]

RESUMO

Caracterização e virulência de isolados de *Metarhizium anisopliae* (Metsch.) Sorok. a ovos de *Deois flavopicta* (Stal) (Homoptera: Cercopidae)

Avaliou-se a variabilidade genética entre 12 isolados de *Metarhizium anisopliae* (Metsch.) Sorok. obtidos de cigarrinhas-das-pastagens, *Deois flavopicta* (Stal) através da análise de isoenzimas, velocidade de germinação dos conídios em meio artificial sólido e virulência ao hospedeiro de origem. Nas análises de isoenzimas não foram detectadas diferenças eletromórficas nos seis sistemas enzimáticos. No entanto, houve variação entre isolados na velocidade de germinação dos conídios e na virulência para *D. flavopicta*. Os valores de tempo de germinação 50% (TG_{50}) variaram de 7:58 a 15:09 (h:min). Os níveis de infecção em ovos na fase de pré-eclosão variaram de 0 a 43,3%. Porém, quando quatro isolados foram testados em ninfas de primeiro ínstar observou-se 35 a 70% de infecção. Não houve correlação entre as características analisadas.

PALAVRAS-CHAVE: Insecta, cigarrinha-das-pastagens, isoenzimas, controle microbiano, fungo entomopatogênico.

The entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorok. (Deuteromycotina: Hyphomycetes) is considered a promising biological control agent for cercopid insects, including pasture spittlebugs of the genus *Deois* and *Zulia* (Conti *et al.* 1980, Alves 1992). Laboratory and greenhouse bioassays testing this fungus against pasture spittlebugs showed virulence higher than 70% (Araújo & D'Aguiar 1975, EMGOPA 1983a, Saldanha *et al.* 1993).

Received 10/21/94. Accepted 10/30/95.
[1]EMBRAPA/CENARGEN, Caixa postal 02372, 70849-970, Brasília, DF.
[2]IMYZA/CICA/INTA, Castelar, Casilla correo 25, 1712 Castelar, Buenos Aires, Argentina.

However, field tests with *M. anisopliae* demonstrated low efficiency, with approximately 5% of infectivity (Ramiro & Cottas 1979, Cottas & Ramiro 1981, EMGOPA 1983b, Carneiro 1988). This low performance is attributed mainly to formulation of the fungus and application methodologies (Alves 1992). Regarding application, there is a poor definition of the timing for introduction of the pathogen, and a lack of information about the more susceptible developmental stages of the host. Phenological and population dynamics studies of the pasture spittlebug *Deois flavopicta* (Stal) indicated that hatching eggs and first instar nymphs of the first generation of this insect, which occur in the beginning of the rainy season each year, would be the best target for the application of entomopathogenic fungi (E.M.G. Fontes *et al.*, not published). In this work we analyzed the virulence of *M. anisopliae* isolates, obtained from adults or nymphs of *D. flavopicta* (Table 1), to hatching eggs of the original host. Some isolates were also tested on first instar nymphs to compare virulence among these developmental stages. Isozyme analysis and conidia germination rate of all isolates were also evaluated in order to understand the relationship between virulence and these two characteristics.

Monoconidial cultures of the isolates were produced and maintained on Sabouraud dextrose agar with yeast extract (40g dextrose, 10g mycological peptone, 10g agar in 1000ml of distilled water, pH 5.6 at 27°C). The conidia were collected after 10 days of cultivation. To determine the germination rate, 50 µl of 10^6 conidia/ml supensions were inoculated on Petri

Table 1. Analysis of germination rate of conidia of *Metarhizium anisopliae* obtained from *Deois flavopicta* on artificial medium and virulence to the original host.

Isolates[1]	Geographic origin	Date	TG_{50}[2] (h:min)	Slope	Mortality(%)[3]	
					Eggs	Nymphs
CG 30	Espírito Santo	NI	15:09 a	26.28	3.3	35
CG 31	Rio de Janeiro	NI	12:46 b	17.08	10.0	ND
CG 58	Mato Grosso	1986	12:43 b	17.58	0.0	ND
CG 45	Brasília/DF	1989	12:25 c	8.68	0.0	ND
CG 42	Brasília/DF	1989	11.43 d	11.80	13.3	ND
CG 51	Brasília/DF	1988	11.32 e	5.98	30.0	ND
CG 102	Brasília/DF	1988	11:05 f	5.71	40.0	ND
CG 49	Brasília/DF	1989	10:54 g	4.87	6.7	70
CG 257	Brasília/DF	1988	10:42 h	6.46	3.3	65
CG 54	Brasília/DF	1986	10:39 h	6.76	20.0	ND
CG 112	Brasília/DF	1988	09:04 i	4.82	43.3	55
CG 29	Manaus/AM	NI	07:58 j	9.00	0.0	ND

[1]CG = CENARGEN/EMBRAPA Collection, Brasília, DF.

[2]Time needed for germination of 50% of the conidia on artificial solid medium. Means followed by the same letter do not differ by Duncan's test at 5% level of probability.

[3]Values are means of three replicates with 10 eggs or 10 first instar nymphs per replicate. No mortality was observed in the controls on the time of evaluation.

NI = not identified.

ND = not determined.

dishes containing the above culture media. A total of 400 conidia (four replicates of 100 conidia) were observed each two hours beginning two hours after inoculation. The data on percentage of germination were used to calculate the TG_{50} (time necessary for germination of 50% of the conidia) using the Probit method. Mycelia needed for biochemical studies were obtained by inoculating 50ml of Sabouraud dextrose broth in 250-ml flasks with a final conidial suspension of 10^6 conidia/ml. Flasks were incubated on a rotary shaker mainteined at 150 rpm and 25°C. After seven days, the mycelia were separated from supernatant by filtering through filter paper (Watman # 1). The mycelial mat was then washed several times in SDW and in a 0,05 *M* Tris-HCL, pH 7.8, buffer to remove residual broth. Mycelia were collected and concentrated by vacuum filtration, weighted, fragmented with liquid nitrogen, and centrifuged at 10.000 g at 4°C for 90 minutes. The supernatant, containing the soluble proteins, was distributed in 1.5 ml tubes and stored at -80°C. The isozymes were separated using isoelectric focusing (IEF) in polyacrylamide gels at 10%, according to the methodology described by Riba *et al.* (1986). Six enxymataic systems were analyzed: alcohol dehydrogenase (ADH), acid phosphatase (ACP), phosphoglucomutase (PGM), glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and glutamate oxalacetic transaminase (GOT), according to staining procedures adapted from Shaw & Prasad (1970).

Eggs close to hatching and first instar nymphs of *D. flavopicta*, obtained from field collected females, were used on the virulence tests. Eggs were inoculated through topic application of 5 µl of conidia suspension (10^8 conidia/ml) with the aid of a micropipette. Three replicates of 10 eggs were used. After the treatment, the eggs were transferred to Petri dishes covered at the botton with a thin layer of wet cotton covered with filter paper. This layer was kept permanently wet. Ten rice seedlings were also added to each Petri dishes to feed the nymphs as the egg hatched. The Petri dishes were incubated at 27°C and 12 hours photophase. After 10 days, the percentage of infected eggs was determined by failure of hatching with mycosis and sporulation. The first instar nymphs were immersed for 5 seconds in a suspension containing 10^8 condia/ml. Three replicates of 10 nymphs were used. After treatment, the nymphs were transferred to Petri dishes, repeating the methodology described above for eggs. After 10 days of the treatment, the percentage of infected nymphs was determined by sporulation of the fungus over the cadaver. Distilled water mixed with Tween 80 at 0.1% was used in the control treatment for both tests.

The enzymatic systems analyzed, although polymorphic for *M. anisopliae* (Riba *et al.* 1986, St. Leger *et al.* 1992), presented homogeneity among *D. flavopicta* isolates used in this study. These results confirm the low variability found among Brazilian isolates of *M. anisopliae* through analysis of isoenzymatic phenotypes (Riba *et al.* 1987, St Leger *et al.* 1992), exoenzymes production (Rosato *et al.* 1981), and pyrolysis-gas chromatography (Messias *et al.* 1983). Using arbitrarily primed analysis PCR, Tigano-Milani *et al.* (1995b) showed high similarity among isolates originally obtained from cercopid insects in Brazil. In the same study, Brazilian isolates obtained from non-cercopid hosts, or from soil, were well separated from the former homogeneous group, probably indicating that the spittlebugs would be acting on the selection of specific genotypes. However, when characteristics better linked to potential of the isolate for biological control agent were analyzed, such as virulence to the original host and germination rate of conidia in artificial media, a high variability among the isolates obtained from the pasture spittlebug, *D. flavopicta*, was observed. The percentage of mortality of *D. flavopicta* eggs caused by *M. anisopliae* varied from 0 to 43%, showing that this stage of development can be susceptible to *M. anisopliae*. First instar nymphs higher susceptibility to *M. anisopliae* than eggs, as the rate of infection varied from 35 to 70% for the four isolates analyzed (Table 1). The isolate CG 49 infected only 6.7% of the eggs, however

the same isolate tested in nymphs showed 70% of infection. Germination of conidia, evaluated through TG_{50} values, varied from 7:59 to 15:09 (h:min) among the isolates studied (Table 1). Although variability was found among the isolates through the analysis of these biological traits, no correlation was observed between virulence and conidial germination. The isolates CG 29 and CG 112 showed capability of fast germination (7:58 and 9:04 h:min, respectively). However, these two isolates showed different behavior in relation to pathogenicity to spittlebug eggs: CG 29 was not pathogenic and CG 112 caused 43.3% mortality (Table 1). The lack of correlation between these parameters was also observed among isolates of another entomopathogenic fungus, *Nomuraea rileyi*, when inoculated on larvae fo *Spodoptera frugiperda* (J.E. Smith) (Tigano-Milani *et al.* 1995a).

These results showed that, for the *M. anisopliae* isolates analyzed, molecular markers such as isoenzymes do not allow separation of isolates based on biological characteristics of interest for biological control. Regarding virulence, eggs of *D. flavopicta* were not so susceptible to *M. anisopliae* as first instar nymphs. Thus, based on the population dynamics of this pest, the selection of isolates virulent to early nymphal stages is indicated, to facilitate the adoption of proper control strategies.

ACKNOWLEDGEMENTS

We thank Bonifácio Magalhães and Edison R. Sujii (CENARGEN/EMBRAPA) for critical review of this manuscript.

REFERENCES CITED

Alves, S.B. 1992. Perspectivas para utilização de fungos entomopatogênicos no controle de pragas no Brasil. Pesq. Agropec. Bras. 27: 77-86.

Araújo, D.O.B. & Z.M.F. D'Aguiar. 1975. Controle biológico das cigarrinhas-das-pastagens. Bol. Inst. Biol. Bahia 14: 1-5.

Carneiro, M. de F. 1988. Eficiência de diferentes cepas de *Metarhizium anisopliae* no controle de *Deois flavopicta*. Pesq. Agropec. Bras. 23: 685-689.

Conti, E., C.L. Messias, H.M.L. Souza & J.L. Azevedo. 1980. Eletrophoretic variations in esterases and phosphatases in eleven wild-type strains of *Metarhizium anisopliae*. Experientia 36: 293-294.

Cottas, M.P. & Z.A. Ramiro. 1981. Flutuação populacional de cigarrinhas-das-pastagens em áreas tratadas e não tratadas com o fungo entomógeno *Metarhizium anisopliae* (Metsch.) Sorokin, 1883. Biológico 47: 255-261.

EMGOPA. 1983a. Efeito de cepas introduzidas e um isolado nativo de *Metarhizium anisopliae* (Metsch.) Sorokin na mortalidade de *Deois flavopicta* (Stal, 1834). Relat. Téc. EMGOPA - 1982, p.99.

EMGOPA. 1983b. Teste de cepas de *Metarhizium anisopliae* para o controle biológico das cigarrinhas. Relat. Téc. EMGOPA - 1982, p.95.

Messias, C.L., D.W. Roberts & A.T. Grefig. 1983. Pyrolysis-gas chromatography of the fungus *Metarhizium anizopliae*: an aid to strain identification. J. Invertebr. Pathol. 42: 393-396.

Ramiro, Z.A. & M.P. Cottas. 1979. Ensaio de campo com diferentes dosagens de *Metarhizium anisopliae* (Metsch.) Sorokin, 1883, visando o controle de *Deois flavopicta* (Stal, 1824) e *Zulia entreriana* (Berg, 1879) em pastagens. Biológico 45: 199-204.

Riba, G., M.L. de Aquino & S.A. Ribeiro. 1987. Polymorphisme chez des souches brèsiliennes de l'Hyphomycète entomopathogène *Metarhizium anisopliae* (Metsch.) Sorokin, inféodées à des Cercopidae. Agronomie 7: 763-768.

Riba, G., I. Bouvier-Fourcade & A. Caudal. 1986. Isoenzymes polymorphism in *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) entomogenous fungi. Mycopathologia 96: 161-169.

Rosato, Y.B., C.L. Messias & J.L. Azevedo. 1981. Production of extracellular enzymes by isolates of *Metarhizium anisopliae*. J. Invertebr. Pathol. 38: 1-3.

Saldanha, L.A., J.O. Pereira & N.M. Silva. 1993. Bioensaios sobre *Deois incompleta* envolvendo uma linhagem selvagem de *Metarhizium anisopliae* (Metsch.) Sorokin e seu mutante morfológico, isoladas em Manaus - Amazonas, p. 317. In Anais Congresso Brasileiro de Entomologia, 14, Piracicaba, 807p.

Shaw, C.R. & R. Prasad. 1970. Starch gel electrophoresis of enzymes: a compilation of recipes. Biochem. Gen. 4: 297-320.

St Leger, R.J, B. May, L.L. Allee, D.C. Frank, R.C. Staples & D.W. Roberts. 1992. Genetic differences in allozymes and in formation of infection structures among isolates of entomopathogenic fungus *Metarhizium anisopliae*. J. Invertebr. Pathol. 60: 89-101.

Tigano-Milani, M.S., M.R. de Faria, R.E. Lecuona, M.R. Sartori, E.Y. Arima & B.M. Diaz. 1995a. Análise de patogenicidade, germinação, e isoenzimas do fungo *Nomuraea rileyi* (Farlow) Samson isolado na região do Distrito Federal. An. Soc. Entomol. Brasil 24: 53-60.

Tigano-Milani, M.S., A.C.M. Gomes & B.W.S. Sobral. 1995b. Genetic variability of Brazilian isolates of the entomopathogenic fungus *Metarhizium anisopliae*. J. Invertebr. Pathol. 65: 206-210.