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Synthesis:

The use of *Metarhizium anisopliae* var. a*cridum* against the grasshopper *Rhammatocerus schistocercoides* in Brazil

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Abstract

The control of grasshoppers in Brazil has been based exclusively on chemical insecticides (fenitrothion and malathion). However, as these products are known to be harmful to the environment, their massive use has caused concerns. In the face of pressure against their use, the development of alternative methods became imperative. Some species of entomopathogenic fungi can supplement or even replace chemical insecticides in the control of grasshoppers. An integrated research project began in Brazil in 1993 with the specific objective of developing bioinsecticides based on entomopathogenic microorganisms, especially fungi, to control grasshoppers. Activities centered in surveys, characterization, production, formulation, and field evaluation. Emphasis was given to developing the fungus *Metarhizium anisopliae* var. *acridum* as the most promising biocontrol candidate. It is now known that this pathogen can be used efficiently in the control of *Rhammatocerus schistocercoides* in Brazil and we are verifying its effects on non-target organisms, including other Orthoptera, Diptera and Hymenoptera.

Key words

Grasshoppers, mycopesticide, fungus, Brazil

Introduction

In past years, native species of grasshoppers have caused severe losses to crop systems in Brazil through seasonal outbreaks determined by environmental conditions. There are at least 23 species of grasshopper considered potential pests. The most serious outbreak occurred from 1984 to 1988 in Mato Grosso State, Central West Brazil, where *Rhammatocerus schistocercoides* (Rehn) invaded approximately 2 million ha. It seems that grasshopper outbreaks are mostly associated with the rainfall regime, especially from August to October, a critical period for the grasshopper cycle (Miranda *et al.* 1996). Outbreaks of different species were also recorded in other regions. In the south, Rio Grande do Sul State, *Rhammatocerus conspersus* (Bruner) invaded *ca* 50,000 ha in 1991/1992 (Cosenza *et al.* 1994). In the northeast, outbreaks of *Schistocerca pallens* (Thunberg) were recorded many times (1888, 1985, 1996). In the last two years, a species complex has attacked pastures in Minas Gerais State during the rainy season, moving to banana orchards nearby during the dry season.

An integrated research project was initiated in Brazil in 1993 at Embrapa (Brazilian Agricultural Research Corporation) Genetic Resources and Biotechnology, with the specific objective of developing bioinsecticides based on entomopathogenic microorganisms,especially fungi, to control grasshoppers. Our activities have included surveys, characterization, production, formulation, and field evaluation. Emphasis has been given to develop the fungus *Metarhizium anisopliae* var. *acridum* as the most promising candidate found as biocontrol agent against grasshoppers in Brazil .

Results

Survey.— In an attempt to find new pathogens, grasshopper-infested areas were surveyed. So far, fungi and nematodes (mermithids) are the only groups of pathogens found infecting grasshoppers in their natural environment. Studies were then concentrated on fungi. Seven isolates of *Metarhizium anisopliae* var. *acridum* and 5 of *B. bassiana* were found infecting *S. pallens* (Rio Grande do Norte, Paraíba) and *R. schistocercoides* (Mato Grosso), respectively. [These isolates are deposited at the Collection of Entomopathogenic Fungi (Embrapa Genetic Resources and Biotechnology)].

Bioassays.— One isolate of *M. anisopliae* var. *acridum*, CG 423, (Moreira *et al.* 1996, formulated in soybean oil + kerosene (5%), showed high virulence against *R. schistocercoides* in the laboratory. This was as compared to other isolates of *M. anisopliae* var. *acridum* (IMI-330189, ARSEF-324), *M. anisopliae* var. *anisopliae* (CG 087), *M. anisopliae* var. *majus* (ARSEF 297) and *B. bassiana* (CG 250, CG 425) (Magalhães *et al.* 1997b). The isolate CG 423 is also highly virulent against the proscopid grasshopper *Stiphra robusta* (Vicentini 1999).

Characterization.— *M. anisopliae* var. *acridum* was initially identified as *M. flavoviride* using arbitrarily primed PCR (Magalhães *et al.* 1997b). However, recent studies have shown that this species is more correctly identified as *M. anisopliae* var. *acridum* (Driver *et al.* 1999). Previous studies using arbitrarily primed PCR (AP-PCR/RAPD) analysis have

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shown only a little genetic variation among isolates of *M. anisopliae* var. *acridum* (Silveira *et al.* 1998). However, Inglis *et al.* (1999) have used telomeric fingerprinting to unambigously differentiate several Brazilian strains of *M. anisopliae* var. *acridum*, as well as strains from Africa and Australia. Using this technique, similarity estimates of telomeric DNA among distinct strains were less than 50%, showing this feature to be highly mutable in this species.

Transformation.— *M. anisopliae* var. *acridum* was used as a transformation model to obtain resistance to the fungicide benomyl by a polyethylene glycol-mediated procedure using a mutant tubulin gene from the fungus *Neurospora crassa*. Transformation frequencies of up to 84 transformants per microgram of transforming DNA were achieved. Benomyl-resistant transformants that could tolerate >30 µg ml^{-1} benomyl were obtained. Southern blot analysis of genomic DNA revealed that the mechanism of genetic transformation was by homologous replacement of the β-tubulin allele (Valadares-Inglis & Inglis 1997).

Infection process.— The development of *M. anisopliae* var. *acridum* in *R. schistocercoides* was investigated. Conidia germinated 12 h after inoculation on fifth instar nymphs, kept at 26-28° C in under 12L:12D photoperiod, following hyphal and appressorial formation before host cuticle penetration. The fungus then continued to develop inside the haemocoel, with formation of typical conidiogeneous cells and conidia 3 d after inoculation. Conidia formed internally, germinated, producing hyphae that penetrated the cuticle from beneath. The pathogen sporulated externally 5-6 d after inoculation (Vicentini & Magalhães 1996). The infection process pattern of *M. anisopliae* var. *acridum* on *S. robusta* and *R. schistocercoides* was very similar (Vicentini 1999).

Effects of M. anisopliae var. acridum infection on food consumption of R. schistocercoides and S. robusta.— Foliage consumption by nymphs and adults of *R. schistocercoides* infected with *M. anisopliae* var. *acridum* was evaluated. Sixth and eighth instar nymphs and female adults consumed on average 85.4, 35.9 and 41.9% of their body weights, respectively. For eighth instar nymphs, the total consumption following 10 d inoculations of 5000 conidia per insect, was 74.5% lower than the consumption of the control insects. For adult females, consumption was 45.6% lower than untreated insects. A strong correlation ($r^2 = 0.998$) between production of fecal pellets by *R. schistocercoides* and food consumption was observed. These results confirmed the high voracity of *R. schistocercoides* and then showed a remarkable adverse effect of the fungus on food intake by infected nymphs and adults (Faria *et al.* 1999). Leaf consumption for *S. robusta* was highly affected by *M. anisopliae* var. *acridum* infection as well. Insects exposed to leaves sprayed with the pathogen, reduced leaf area consumed from 64% on the 1st day to 0% on the 4th day (Vicentini 1999).

Compatibility of M. anisopliae var. acridum and sub-lethal doses of chemical insecticides against R. schistocercoides.— In a study as yet unpublished, we investigated whether the speed of action of *M. anisopliae* var. *acridum* could be improved by adding sublethal doses of chemical insecticides to the fungal formulation. First, the effect of different insecticide concentrations (5-5000 ppm) on fungus development was determined. Second, we assessed the effects of *M. anisopliae* var. *acridum* and chemicals, isolated and in association, on *R. schistocercoides* nymphs. Low doses of teflubenzuron, chlorpyriphos, fenitrothion, malathion and deltamethrin did not affect either fungal germination or radial growth on solid medium. Inhibitory concentrations (IC_{50}) were, respectively, 3596, 2414, 986, 378 and 275 ppm for germination and 4729, 33, 28, 344 and 28 ppm for radial growth. Bioassays showed that topical inoculation of 3 µml of oil suspension (1.5×10^6 conidia / µml) in association with sublethal concentrations of fenitrothion (25-250 ppm) on *R. schistocercoides* was able to change onset of mortality. Mortality onset went from 96 h (treatment with fungus alone) to 48 h (treatment with conidia + 25 ppm of fenitrothion) or even to 24 h (treatment with conidia + 50 or 250 ppm of fenitrothion). Mortality levels observed indicated a synergistic effect between *M. anisopliae* var. *acridum* and this chemical insecticide (S. Xavier-Santos, B.P. Magalhães & M.R. Faria, unpublished).

Production and formulation.— Substrate and its water content in association with temperature can affect conidial production by Hyphomycetes. The manipulation of these variables to optimize conidial production of *M. anisopliae* var. *acridum* was investigated. Best results were obtained when the fungus was grown using equal parts of parboiled rice and water (1:1, weight:volume). Aiming at the mass production of the pathogen, a cooperative project was developed with a small unit designed to produce fungi for use in biological control of insects and plant diseases in São José do Rio Claro County (Mato Grosso State). This was successful in producing the conidia needed for field experiments in Mato Grosso in 1998.

Rapid inactivation of conidia by solar radiation may be a problem in some areas. The use of sunscreens in conidial oil formulations was considered to increase the persistence of the pathogen in the field. Different sunscreens (cinnamic acid ethyl ester, tinopal, congo red and melanin) were tested in baits (oat flakes). Toxicity to *M. anisopliae* var. *acridum* conidia, palatability to the grasshopper *R. schistocercoides* and protection of conidia against solar radiation were assessed. At the concentrations tested, these products did not affect germination and palatability of the fungus. However, there was no protective effect on conidia against solar radiation, although conidia formulated as baits lasted longer (Faria *et al.* 1997).

Conidial viability in oil formulation.— A simple technique to verify the viability of conidia formulated in oil was developed (Magalhães *et al.* 1997a). Blocks (1.0 X1.0 X 0.2 cm) of potato dextrose agar medium (potato 170 g, dextrose 20 g, agar 20 g, water 1 liter) were placed on sterile glass slides. Conidia germinated at similar percentages when formulated in oil, with or without kerosene, plated on nutrient medium and covered with a coverslip. At 18 h, germination

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was very high (> 97%) and comparable to that estimated by the conventional aqueous suspension method. However, when the oil formulation was plated and no coverslip used, germination was poor and very difficult to quantify, since nongerminated conidia were frequently confounded with small air bubbles. The growth rate of *M. anisopliae* var. *acridum* formulated in oil or in oil + kerosene, under a coverslip, was comparable to the growth rate in aqueous suspension with no coverslip (conventional method).

Field tests.— The efficacy of a mycoinsecticide formulated in vegetable oil has been tested against *R. schistocercoides* several times since 1995. In 1998, a set of experiments was conducted in the Chapada dos Parecis region, a permanent zone of outbreaks for this pest in Mato Grosso State. Experiments were performed in zones of natural vegetation, against bands in the third nymphal instar. Three nymphal bands were treated with a mycoinsecticide formulation based on conidia of the entomopathogenic fungus *M. anisopliae var. acridum*, strain CG 423 (dosage 2×10^{13} conidia/ha). Three non-treated bands were used as control. The application was made with the aid of a hand ultra-low-volume (ULV) sprayer adjusted to deliver 21/ha of the formulation, each litre containing 1×10^{13} conidia. Treatments were limited to areas occupied by the band and within 5-10 m of its immediate borders. The efficacy was evaluated through band survival after treatment (grasshopper numbers, surface, density, behavior and daily movement of the band), letting the insects move freely in their natural environment. Insects maintained in the laboratory were also regularly surveyed to estimate the infection rate. Results from both field and laboratory showed a clear effect of the product 10 d after treatment. At 14 days post-spraying, mortality caused by the mycoinsecticide in the field was approx. 88% (Magalhães *et al.* 2000).

In 1999, another experiment was performed aiming to reduce the dose utilized the year before. Conidia formulated in a racemic mixture of soybean oil and kerosene were sprayed under field conditions using an ULV hand-held atomizer, adjusted to deliver 2.9 l/ha. Bands composed of 2nd instar nymphs were treated with either 5.0×10^{12} or 1.0×10^{13} conidia / ha. Number of insects in each band was estimated at day one following spraying and by the end of the field trial (15-16 d post-treatment). Reductions in population size reached an average of 65.8% and 80.4% for bands treated with the higher and lower dosage respectively. For both dosages, total mortality rates of insects collected at 2 d postapplication, and kept in cages for 14 d under lab conditions, showed no significant differences as compared to that obtained with insects collected immediately after spraying ($p \geq 0.282$). When healthy insects were fed native grasses sprayed at the field with 1.0×10^{13} con./ha, mortality levels observed at 2 and 4 d postapplication were not affected when compared to day zero ($p \geq 0.050$) (Faria *et al.* 2001).

In 2000, we studied the effects of *M. anisopliae* var. *acridum* on non target arthropods, with special reference to orthopterans. The experimental protocol consisted of 4 blocks of habitat, each one of them possessing two 0.49 hectare parcels (70 X 70 m). In each block, one of the parcels was sprayed with a dosage equivalent to 4.2×10^{12} viable con. /ha, and in the neighboring parcel no application was made. Immediately before and in regular intervals after spraying (4, 7, 10, 13, 16 and 19 d), the densities of nymphs and adults of non target orthopterans was evaluated in each parcel by visual estimations made over fifty 1-m^2 squares. Seven days after application (DAA) it was possible to observe a decrease in the density of nymphs in the sprayed parcels. Although significant differences were observed only at 16 DAA ($p = 0.015$), the density of nymphs during the whole period of the experiment underwent a 28.8% reduction in those parcels sprayed with the fungus, and an increase of 4.1% in the control parcels. For orthopteran adults, the effect of the mycoinsecticide was more obvious at 13 DAA. Although this evaluation was the only one in which the density of adults was significantly lower than in the parcels sprayed with the fungus ($p = 0.021$), the reduction in adult densities in the sprayed parcels was of 59.7%, whereas in the control an increment of 5.8% was observed. The results demonstrate that the biopesticide for the control of *R schistocercoides* can affect populations of non-target orthopteroids. Data analysis regarding other insect orders, from an evaluation performed over two 4-ha parcels, are in progress. Preliminary results suggest a negligible lethal effect, with the likely exception of some Diptera from the family Asilidae.

Concluding remarks.— The implementation of entomopathogenic fungi as bioinsecticides against grasshoppers in Brazil is greatly limited by the lack of a consistent production system, short shelf-life, and their slow action in killing the host. Effort is being directed to optimize the production system for *M. anisopliae* var. *acridum* and to increase its storage capacity at room temperature. The slow action in killing the host is minimized by the apparent reduction in mobility and food consumption presented by the infected insects, and by the fact that young nymphs of *R. schistocercoides* usually occur in natural vegetation instead of cultivated areas. Another problem is that *R. schistocercoides* is univoltine. This allows only one field trial with small nymphs per year, in the period between November and December. The lack of serious grasshopper problems in Brazil in recent years has hampered the development of research in this area, since funds were reduced. The isolate CG 423 of *M. anisopliae* var. *acridum* is highly virulent against the grasshoppers *R. schistocercoides* and *S. robusta*, and probably other species, and the availability of this good candidate as a mycoinsecticide may be useful in the future.

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