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A GRANULAR FORMULATION OF *Nomuraea rileyi* Farlow (Samson)

FOR THE CONTROL OF *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE)

Domenico Pavone, Mayri Díaz, Lesbia Trujillo and Blas Dorta

SUMMARY

A granular formulation of the entomopathogenic fungus *Nomuraea rileyi* (Farlow) Samson was evaluated against *Spodoptera frugiperda* (Lepidoptera: Noctuidae). The formulation consisted of 1mm particles of defatted corn germ (DCG) containing 10^7 conidia/g. This preparation protected the conidia against UV ra-

diation and killed 80% of *S. frugiperda* larvae in laboratory bioassays. It was shown that the fungus used DCG as a substrate for growth and sporulation, creating foci for further infection. This strategy has great potential for the formulation of fungal biocontrol agents, especially those with a high growth rate.

FORMULACIÓN GRANULADA DE *Nomuraea rileyi* Farlow (Samson) PARA EL CONTROL DE *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE)

Domenico Pavone, Mayri Díaz, Lesbia Trujillo y Blas Dorta

RESUMEN

Una formulación granulada del hongo entomopatógeno *Nomuraea rileyi* (Farlow) Samson fue evaluada contra *Spodoptera frugiperda* (Lepidoptera: Noctuidae). La formulación consistió de partículas de 1mm de diámetro de germen desgrasado de maíz (GDM) inoculadas con 10^7 conidias/g. La preparación protegió a las conidias de la radiación UV y eliminó al 80% de

la población de larvas de *S. frugiperda* en bioensayos de laboratorio. Se demostró que el hongo es capaz de utilizar el GDM como un sustrato de crecimiento y esporulación creando nuevos focos de infección. Este tipo de estrategias posee gran potencial para la formulación de agentes fúngicos de biocontrol, especialmente aquellos con alta tasa de crecimiento.

FORMULAÇÃO GRANULADA DE *Nomuraea rileyi* Farlow (Samson) PARA O CONTROLE DE *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE)

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RESUMO

Uma formulação granulada do fungo entomopatogênico *Nomuraea rileyi* (Farlow) Samson foi avaliada contra *Spodoptera frugiperda* (Lepidoptera: Noctuidae). A formulação consistiu de partículas de 1mm de diâmetro de germen desengordurado de milho (GDM) inoculadas com 10^7 conidias/g. A preparação protegeu às conidias da radiação UV e eliminou 80% da popu-

lação de larvas de *S. frugiperda* em bioensaios de laboratório. Demonstrou-se que o fungo é capaz de utilizar o GDM como um substrato de crescimento e esporulação criando novos focos de infecção. Este tipo de estratégias possui grande potencial para a formulação de agentes fúngicos de biocontrole, especialmente aqueles com alta taxa de crescimento.

Introduction

Entomopathogenic fungi have great potential for integrated pest management programs due to their specificity, mode of action and ease of application. *Nomuraea rileyi*

(Farlow) Samson is an entomopathogenic fungus found in several countries, including Brazil and Venezuela. This fungus attacks important caterpillar pests of soybean and corn such as *Spodoptera frugiperda* Smith (Lepidoptera:

Noctuidae), causing epizootics (Ignoffo *et al.*, 1976; Piñango *et al.*, 2002). Environmental conditions (solar radiation, humidity, etc) greatly affect the microorganisms, decreasing their field viability and persistence. Current research

has thus been focused on minimizing the effect of these conditions to increase fungus survival and effectiveness.

Biocontrol agent formulations are powerful tools for achieving this goal (Auld 1992; Goettel and Roberts,

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1992; Pereira and Roberts, 1991; Rodham *et al.*, 1999; Lacey *et al.*, 2001). Liquid formulations with added oil improve fungus field performance (Prior *et al.*, 1988; Bateman *et al.*, 1993; Ibrahim *et al.*, 1999; Luz *et al.*, 1999; Maiga *et al.*, 1999; Smith *et al.*, 1999; De Courcy *et al.*, 2000; Inyang *et al.*, 2000) and additives such as chemical UV filters can protect fungi from UV radiation (Inglis *et al.*, 1995; Leland *et al.*, 2001).

Granular formulations have also been used as an alternative for improving the efficacy of several biocontrol agents. Fungi have been prepared as granules containing dry mycelia or conidia formulated as contact baits. These granules have been used as insecticides (Schwarz, 1995; Shah *et al.*, 1999, 2000), mycoherbicides (Walker and Connick, 1983; Connick *et al.*, 1998), nematocides (Stirling and Smith, 1998) and plant pathogen antagonistic fungi (Lewis and Larkin, 1998). This type of formulation may act as a solid culture medium for fungal growth in the field (Hua and Feng, 2003) or simply as a vehicle for infection (Stirling and Smith, 1998; Maniania, 1993). Generally, particle size in formulations ranges from 0.3-1mm; thus, dry mycelium or conidia inside the granules are protected from sunlight. Additionally, granules can act as insect attractants increasing their contact with the fungus (Leland, 2001).

This study describes the development of a granular formulation based on *N. rileyi*, which could function both as a growth culture medium and as an aid to fungal sporulation in the field, thus creating foci for further infection, leading to improved efficiency for the control of *S. frugiperda*.

Materials and Methods

Biological material

N. rileyi isolate LPFIBE-3, supplied by the *Centro Venezolano de Colecciones de*

Microorganismos (CVCM), is a strain originally obtained from a mummified field-collected larva of *S. frugiperda* in a corn plantation in Guárico State, Venezuela. The fungus was maintained on DYPA agar slants containing 5g dextrose, 1g peptone, 2g yeast extract, 1g NH₄NO₃, 1g K₂HPO₄, 0.5g MgSO₄·7H₂O, 0.01g FeCl₃·6H₂O and 16g agar per liter. *S. frugiperda* larvae were obtained from a population reared in the laboratory as described elsewhere (Parra, 1986).

Fungal cultures and production of conidia

N. rileyi was cultured in 500ml cylindrical screw-capped glass bottles of 10cm bore. Bottles containing 100ml of DYPA medium and allowed to solidify in a flat position were inoculated with 0.5ml of a conidia suspension of *N. rileyi* at a concentration of 10⁶ conidia/ml. Conidia were spread on the surface and the bottles were incubated at 25 ±2°C for two weeks, under continuous artificial light. After incubation, conidia were harvested by adding 100ml of sterile 0.1% Tween 80 in distilled water and shaking by hand. The concentration of conidia was determined in a Neubauer haemocytometer.

Granular formulation

Granules consisted of defatted corn germ (DCG), supplied by Empresas Polar (Promasa), Turmero, Aragua

State, Venezuela. This by-product is an excellent solid substrate for culturing *N. rileyi* (Pavone, 2003). Auto-claved DCG was inoculated with *N. rileyi* conidia at a concentration of 10⁷ conidia/g dry matter, and the preparation's water content was adjusted to 50% on a wet weight basis. The preparation was aseptically extruded through a cribbed plaque using a hand-operated mincing-type machine to obtain 2mm diameter filaments. These filaments were finally broken into 2-3mm-long fragments which were packed in glass columns of 2.5cm bore and aseptically air-dried. Filtered sterilized air was pumped through the columns at a rate of 0.3l·h⁻¹·g⁻¹ wet matter.

Incubation of granules at varying relative humidities (RH)

For hydration at various RH values, saturated solutions of different salts were used (Table I). The solutions (150ml each) were prepared separately in 600ml plastic containers and autoclaved at 120°C for 15min. Samples (1g) of dry granular formulation were placed in 5ml sterilized plastic cups and aseptically transferred to the containers, which were then sealed and incubated during 50h at 25 ±2°C.

Water activity and water content

Water activity (a_w) of granules at different degrees of

hydration (w_c) was measured at 25°C with a water activity analyzer Aqualab CX-2, (Decagon Devices Inc., Pullman, WA, USA). Water content was determined by an LJ-16 humidity analyzer (Mettler-Toledo AG, Greifensee, Switzerland).

Hydration of granules to promote fungal sporulation

Immediately after the drying process, 1g samples of dry granular formulation were placed in 30ml sterilized plastic containers and incubated until hydration at 25 ±2°C in a room saturated with water vapor under continuous artificial light. Incubations were carried out for 12 days, after which conidial yield was determined. Sporulated granules were re-suspended in 5ml 0.1% Tween 80/g of initial dry matter and conidia were counted as described above.

UV assay

The granular formulation was exposed to UV radiation using a UVLMS-38 lamp (UVP®; Ultra-violet Products, Upland, CA, USA). Three wavelengths were used in independent experiments: 254nm (UV-C), 302nm (UV-B) and 365nm (UV-A) at intensities of 250, 1600 and 2500µW·cm⁻², respectively, adjusted with a UVX radiometer (Ultra-violet Products, Upland, CA, USA). The granular formulation was exposed to UV radiation in open Petri dishes for 15min with periodic agitation.

TABLE I
WATER ADSORPTION KINETICS OF THE GRANULAR FORMULATION AT DIFFERENT RELATIVE HUMIDITY VALUES

Salt / RH (%) at equilibrium	Weight (g)						Final a _w	Final water content (%)
	Initial	after 12h	after 24h	after 36h	after 48h	after 60h		
LiCl / 11	1.03	1.05*	1.05	1.05	1.05	1.05	0.127	2.31
K ₂ CO ₃ / 43	1.04	1.09	1.09	1.1*	1.1	1.1	0.447	5.8
NaCl / 75	1.02	1.16	1.16	1.17*	1.17	1.17	0.753	9.72
KCl / 84	1.05	1.22	1.24	1.25*	1.25	1.25	0.848	11.5
K ₂ SO ₄ / 97	1.01	1.24	1.32	1.36	1.42*	1.42	0.968	29.08
H ₂ O / 100	1.03	1.3	1.42	1.47	1.54	1.56*	0.986	35.77

* Samples reached constant weight.

Bioassay

Forty second instar *S. frugiperda* larvae per treatment were maintained individually in 30ml plastic containers. One gram of the granular formulation was placed in each container and larvae were fed on discs (2cm diameter) of *Ricinus communis* L. leaves. Heat inactivated granules or alternatively leaf discs submerged in a suspension of 10^7 conidia/ml were used as controls. The bioassay was checked daily and the number of dead larvae for each treatment was noted.

Statistical analysis

Mean lethal times 50 and 95 (LT_{50} and LT_{95}) were estimated by Probit analysis using the Probit Analysis Program (Raymond, 1985). Differences between treatments were determined by comparing confidence levels given by the Probit analysis. The best fit isotherm curve was calculated using the software Curve Expert version 1.37.

Results and Discussion

Water relations of granules

The aim of the design of the granular formulation was to produce a solid culture medium for the promotion of sporulation of *N. rileyi* in the field. One of the most important requirements to accomplish this is to provide an adequate water supply to the granules. The water may come from rain, irrigation or, as in this study, water vapor from the atmosphere. The experiments were carried out at various relative humidity values in order to measure the sporulation response of *N. rileyi* on the granules. Water availability for the growth of the fungus on the granules is more dependent on water activity (a_w) than water content (w_c) *per se*, a_w being the relation between the vapor pressure of the granule-water mix and the vapor pressure of pure water. Under equi-

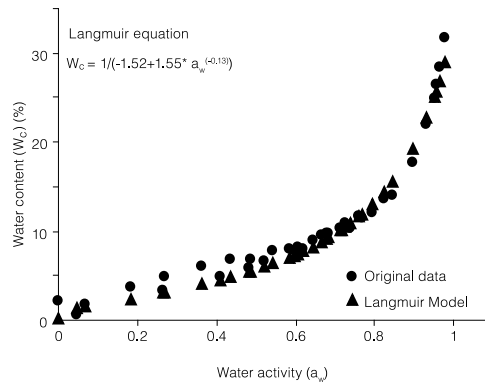


Figure 1. Granular formulation water adsorption isotherm. Samples were incubated in humid chambers saturated with water vapor at 25°C.

librium conditions $a_w/100 = \%RH$. The water adsorption isotherm of the granular formulation, that is, the relation between w_c and a_w at a constant temperature, was determined with non-inoculated granules (Figure 1).

The relationship between w_c and a_w is best described by the Langmuir model (Langmuir, 1918), which was statistically validated by Fowler (1935). Data analysis led to $w_c = 1/(-1.52+1.55 \cdot a_w^{(-0.13)})$ to describe the isotherm (correlation coefficient = 0.99). A minimal water content ($w_c = 30\%$ on a wet-weight basis) is required to reach maximum water availability ($a_w = 0.999$). This value was reached at about 40h when the dry granules were incubated at 100% RH (Figure 2). Filamentous fungi require high levels of a_w for growth and sporulation on solid media (Dorta *et al.*, 1990). An optimal a_w value of 0.977 for the growth of *N. rileyi* was determined in our laboratory (unpublished data), which corresponds to a w_c value of ~30%, according to the adsorption isotherm shown in Figure 1. Based on these results, it can be assumed that under conditions

of 100% RH in the field, at least 40h are needed to reach the minimal w_c needed for growth and sporulation of *N. rileyi* on the granules.

However, as 100% RH is not always reached in the field, it is important to identify the ability of granules to absorb water at lower RH values. Water relations using saturated solutions are shown in Figure 1. RH conditions (11-100%) were characteristic of each saturated salt solution at equilibrium. Thus for each RH condition, the granules reached a constant w_c value whose magnitude agreed with the corresponding adsorption isotherm.

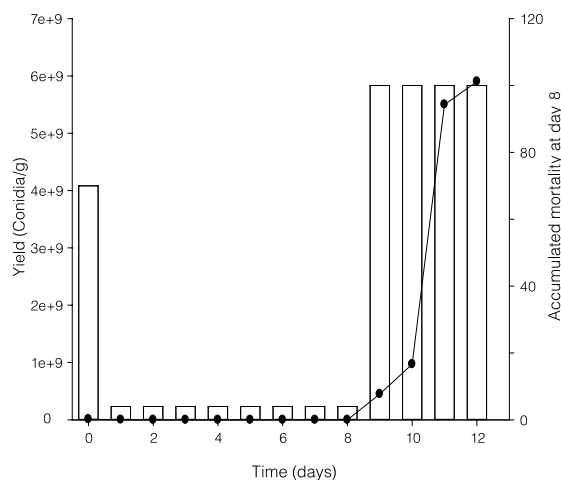


Figure 3. Sporulation of *N. rileyi* on granules (curve) and mortality of *S. frugiperda* larvae exposed to these granules at different levels of sporulation (bars). Forty plastic containers, containing one gram of dry granules were transferred daily for 12 days to a humid chamber (RH=100%) allowing different degrees of sporulation. At day 12, yield (conidia/g) was determined and one larva was placed into each flask (240 in total). Mortality was checked on day 8.

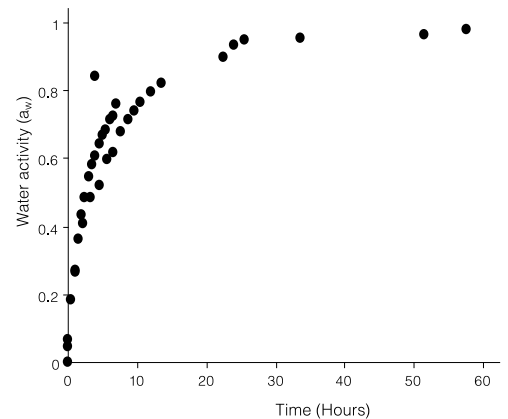


Figure 2. Granular formulation water adsorption kinetics. Samples were incubated in humid chambers saturated with water vapor at 25°C.

The results shown in Table I indicate that under different RH conditions granules absorb water until equilibrium is reached, which occurs sooner at lower RH values. At equilibrium, the granules stop adsorbing water; thus, at RH values lower than 97% granules will not be able to reach the minimal w_c (30% on a wet-weight basis) required to support the growth and sporulation of *N. rileyi*. However, at higher RH values (>97%) the water necessary for fungal growth is appropriately supplied.

Fungal growth and sporulation on granules

The ability of *N. rileyi* to grow and sporulate on the formulated granules was demonstrated under appropriate conditions (100% RH; Figure 3). Sporulation begins on day 9, reaching a maximum yield of 6×10^9 conidia/g dry matter on day 12. Taking into account the initial conidial concentration in the granules (10^7 conidia/g dry matter), it was possible to increase the inoculation rate by 600 times using this method. Values of 6.5×10^8 conidia/g were used in a granular formulation of *M.*

anisopliae for the control of the lesser grain borer *Rhyzopertha dominica* (Batta, 2005) and a residual effect for both the granular and liquid formulations was reported. Figure 3 also shows the effect of sporulating granules on the mortality of *S. frugiperda* larvae. Indeed, after 9 days of hydration, incubated granules were able to cause 100% mortality.

The supplementation of granular formulations with carbon and nitrogen sources has been proposed to enhance sporulation of fungi, although the establishment of cost-benefit ratios is necessary before including these substances (Shah *et al.*, 1999). In the present granular formulation, conidia production per gram was not significantly different from preparations based on GDC alone or those supplemented with sugar cane molasses and/or corn steep liquor (data not shown).

Granular formulations have been prepared with the fungi *Arthrotrichum dactyloides* and *Verticillium chlamydosporium*, the latter always having a less prolific growth than the former (Stirling and Smith, 1998). This fact has important field implications since granules may behave in two ways, as infection foci and as agents for the multiplication of the inoculum. *N. rileyi* is a slow-growing fungus requiring 10-12 days to complete growth and sporulation (Figure 3). This makes the growing process a slow one, increasing the probability of granule contamination. However, this strategy seems to be promising with faster-growing fungi such as *Metarhizium anisopliae* (Metschnikoff) So-

rokin, *Beauveria bassiana* (Balsamo) Vuillemin and *Trichoderma harzianum* Rifai. Indeed, granular formulations of *Trichoderma* spp. have been prepared using vermiculite and wheatbran without aseptic conditions, allowing the proliferation of the biocontrol agent (Lewis and Lumsden, 2001).

Effect of UV radiation on the virulence of the formulation

Bioassays were carried out to evaluate the protective effect of the granules against UV radiation. Figure 4 and Table II show the effectiveness of the granular formulation for killing *S. frugiperda* larvae. The results indicate that the granular formulation was able to reduce 75% of the larval population, compared to unformulated liquid conidia. In this case, conidia were spread on the surface of *R. communis* leaf discs promoting contact with the larvae. In addition, a large number of conidia were ingested by the larvae as they fed on leaf discs. It is important to emphasize that in this case the granular formulation was not yet colonized by the fungus and mortality was therefore due exclusively to conidia on the surface of the granule. Additionally, most conidia were inside the granule matrix, and thus not in direct contact with the larvae. Thus, the number of acces-

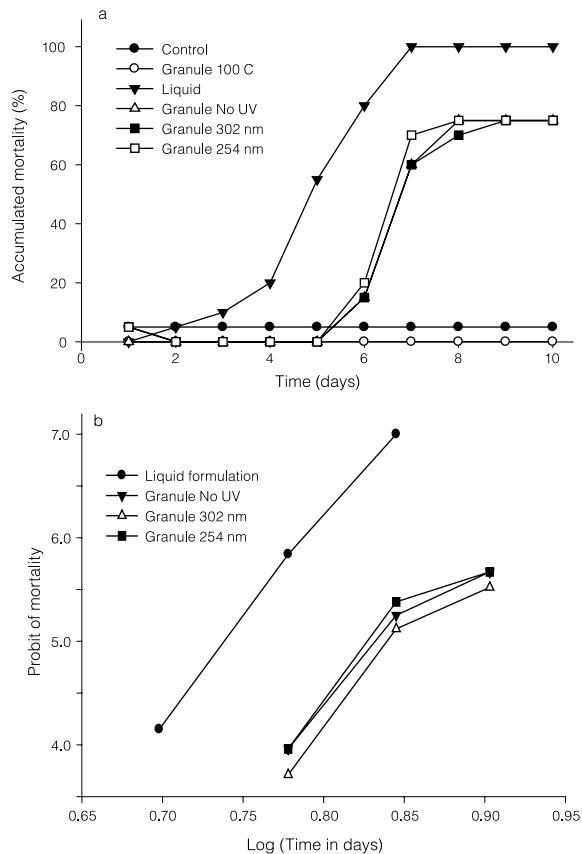


Figure 4. Accumulated mortality (a) and Probit analysis (b) of *S. frugiperda* larvae after application of the granular formulation, with and without UV radiation, and a liquid suspension of *N. rileyi* conidia.

sible conidia in the granular formulation was less than on the leaf discs coming from the aqueous formulation. Granular formulations of *B. bassiana* (Maniania, 1993) and *M. anisopliae* (Ekesi *et al.*, 2005) have been evaluated. These formulations were more efficient than spray applications of aqueous and oily aqueous formulations probably due to their greater persistence in the field.

LT₅₀ and LT₉₅ were not significantly different between granules exposed and not exposed to UV radia-

tion (Figure 4; Table II). These results point to the protective effect of the formulation on the *N. rileyi* conidia from UV radiation. The irregular topography of the granule could act as a physical barrier to UV radiation; as a result, most conidia should be protected. However, this experiment did not determine how many conidia on the surface of the granule were affected by UV radiation, which depends on the surface portion exposed to UV radiation. It is clear that the conidia inside the granules should remain alive, because they were not exposed to UV radiation. It is also important to emphasize that the use of granular formulations based on CDG may have certain limitations, since mycotoxigenic fungus such as *Aspergillus flavus* Link: Fries, commonly found on corn fields, could also proliferate on this substrate (Lewis, 2001). The importance of determining the impact of granules on corn mycotoxin levels is obvious. The high capacity of the granules to cause mortality of *S. frugiperda*, the generation of infective foci and the UV-protection exerted show the potential of this granular formulation of *N. rileyi* for the biocontrol of this insect pest. It is important to emphasize, however, the importance of the implementation of field trials to probe the effectiveness and safety of the formulation under these conditions, which is the focus of our current research.

TABLE II
STATISTICAL ANALYSIS OF DATA REPRESENTED IN FIGURE 4

Treatment	LT ₅₀	Confidence Level	LT ₉₅	Confidence Level
Granule No UV	7.15 ± 0.48 a	6.43 <LT ₅₀ <7.38	9.98 ± 1.6 a	8.20 <LT ₉₅ <12.36
Granule 302nm	7.01 ± 0.14 a	6.72 <LT ₅₀ <7.7	8.86 ± 0.5 a	8.40 <LT ₉₅ <12.87
Granule 254nm	6.94 ± 0.03 a	6.50 <LT ₅₀ <7.46	9.53 ± 0.41 a	8.26 <LT ₉₅ <12.57
Liquid	5.46 ± 0.04 b	5.18 <LT ₅₀ <5.72	6.77 ± 0.31 b	6.08 <LT ₉₅ <7.32

LT₅₀ and LT₉₅ identified with the same letter are not significantly different between treatments.

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