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ORIGINAL ARTICLE

Field evaluation of entomopathogenic fungi formulations against *Rachiplusia nu* (Lepidoptera: Noctuidae) in soybean crop

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Abstract

Rachiplusia nu (Lepidoptera: Noctuidae) is the main soybean plague in Argentina. The main strategy employed to control this pest is chemical control, applying different chemical groups regardless of their harmful effects on the environment and human health. Different biological products using entomopathogenic fungi have been developed and are commercially available to control different insect pests worldwide. The objective of this work was to develop and apply, under field conditions, different fungal formulations using entomopathogenic fungi to control *R. nu* larvae. The mortality percentages in bioassays of *R. nu* larvae treated with different colonies of fungal entomopathogens ranged between 86.6 ± 8.4% for *Beauveria bassiana* (LPSc 1098) and 56.6 ± 4.2% for *Metarhizium anisopliae* (LPSc 907). Under laboratory conditions using fungal formulations of *B. bassiana*, the formulation 4 (LPSc 1086) exhibited the highest mortality percentage (100%), followed by formulation 5 (LPSc 1098), 97 ± 1.3%. Under field conditions, larval mortalities were 82.4 ± 5.56% for formulation F4 and 61.8 ± 7.5% for formulation F5. The results obtained in this work indicate that although a greater number of tests under field conditions with the fungal formulation F4 are necessary, the results obtained in this work allow speculating that it is possible to use this fungal formulation under field conditions to control *R. nu*.

Keywords: bioinsecticide, biological control, crop protection, insect pests

Introduction

Rachiplusia nu (Guenée) (Lepidoptera: Noctuidae) is a polyphagous species considered to be a potential pest of many crops with high commercial value including aromatic and oleaceous plants, such as sunflower (*Helianthus annus* L.), soy [*Glycine max* (L.) Merrill], alfalfa (*Medicago sativa* L.), cotton (*Gossypium hirsutum* L.), beans (*Phaselous vulgaris* L.), linen (*Linum usitatissimum* L.), and tobacco (*Nicotiana tabacum* L.) (Artigas 1994; Pereyra 1995; Pereyra 1998; Pastrana *et al.* 2004; Betancourt and Scatoni 2006; Navarro *et al.* 2009). This lepidopteran is commonly found in Argentina, Bolivia, Peru, Brazil, Chile, and Uruguay (Barrionuevo *et al.* 2012). During the last decade, it has emerged as the main soybean pest in Argentina due to the great economic losses it has caused (de Freitas Bueno *et al.* 2011).

The most valuable crop in South America is soybean (*Glycine max* L.). Argentina, Brazil, and Paraguay alone accounted for more than half of the global soybean production in the 2016/2017 cropping season (USDA 2018). Several insect pests attack this crop causing important economic losses. For this reason, minimizing insect damage is a main concern (Jakubowicz *et al.* 2019). *Rachiplusia nu* is a pest with many natural enemies, such as predators, parasitoids, and even fungi. However, farmers opt for chemical control, which can be done by applying different chemical groups, including pyrethroids, phosphorous, and diamides.

At the beginning of the 70's, the production and consumption of agrochemicals increased considerably, especially in grain-producing countries, increasing exponentially the risks and harmful effects on human health and the environment. Furthermore, recent problems regarding mortality and the negative impact of chemical insecticides on bee populations have been reported (Schuhmann *et al.* 2022). In this sense, there is an urgent need to establish alternative methods for insect control.

Entomopathogenic fungi are naturally found in agroecosystems and these microorganisms are capable of controlling insect pests (Vega *et al.* 2012). As biocontrol agents, entomopathogenic fungi exhibit some advantages over the traditional use of chemicals; they present high specificity, easy natural dispersal, *in vitro* culture with maintenance of pathogenicity and safety for vertebrates (Goettel *et al.* 2010). Different biological products using fungi have been developed and are commercially available to control different insect pests worldwide (Faria and Wraight 2007; Nazir *et al.* 2019). In Argentina, there is no history of application of bioinsecticides based on entomopathogenic fungi in extensive crops. However, other countries in Latin America, like Brazil and Colombia, have formulated and tested several bio-inputs (Faria and Wraight 2007; Mascarin *et al.* 2019). Thus, the aim of this work was to develop and apply, under field conditions, different fungal formulations using entomopathogenic fungi to control *R. nu* larvae, the main lepidopteran pest of soybean crops.

Materials and Methods

Insect rearing

Rachiplusia nu individuals were acquired from AgIdea (Agricultural Innovation Applied Research), where colonies have not been exposed to insecticides for several generations. A laboratory colony was established, using a semi-synthetic diet to feed larvae (Greene *et al.* 1976). Larvae were placed in a raising chamber under controlled conditions (25 ± 0.5°C, 75% RH, and 16/8 h L/D photoperiod). For the pathogenicity assays third stage larvae (L3) were used

Fungal colonies

The fungal colonies were selected from the culture collection of the Spegazzini Institute for their entomopathogenic capacities towards other insect pests (Pelizza *et al.* 2012 a, b). The colonies employed were: LPSc 1067 (GenBank accession number KF500409), LPSc 1082 (GenBank accession number KJ7722495), LPSc 1098 (GenBank accession number KT163259), LPSc 1086 (GenBank accession number MG712626), LPSc 1156 (GenBank accession number MG712627), LPSc 1225 (GenBank accession number MG012790), LPSc 1226 (GenBank accession number MG012791), LPSc 1227 (GenBank accession number MG012792) of *Beauveria bassiana*, LPSc 907 (GenBank accession number KJ772494) of *Metarhizium anisopliae,* and LPSc 963 (GenBank accession number KT163258) of *M. rileyi*. The fungal colonies were cultured on potato dextrose agar (PDA) medium (Britania S.A., Buenos Aires, Argentina) for 10 days at 25°C in the dark. The conidia were obtained using sterile scrapers and posteriorly placed in tubes with 0.01% (v/v) polyoxyethylene sorbitanmonolaurate (Tween 80®; Merck) that were vortexed for 2 min, and filtered through four layers of sterile muslin. The solution concentration was adjusted to 1×10^8 conidia · ml^{-1} utilizing a Neubauer hemocytometer. The viability of the conidia from each isolate used in the test was determined after 24 h as described by Inglis *et al.* (1996) with a suspension of 1×10^4 conidia · ml^{-1}.

Pathogenicity assays

The pathogenicity assay was performed to evaluate the mortality of *R. nu* third instar larvae using the above-mentioned fungal colonies. Larvae were sprayed individually with 300 µl of each treatment using a glass sprayer (discharge rate of 0.10 ± 0.02 ml) and posteriorly settled in 30 cm^3 plastic containers with an artificial diet provided *ad libitum*. Each treatment consisted of three replicates (on different days) of ten individuals and a control group. Control larvae were inoculated using Tween 80® 0.01% (v/v) alone. The larvae were placed in a climatic chamber under controlled conditions at 25 ± 0.5°C, 75% RH, and 16/8 h L/D photoperiod. According to Lacey and Brooks (1997), larval mortality was registered daily for 10 days; dead individuals were surface-sterilized and placed in humid chambers at 25°C in the dark to promote fungal growth. Mycosis was confirmed by microscopic examination of the dead larvae. Significant differences in mortality percentages were assessed using ANOVA and compared *a posteriori* with a Tukey test ($p = 0.05$) using the statistical software Infostat version 2011 (Di Rienzo *et al.* 2011).

Liquid formulation development

When preparing a mycoinsecticide formulation, a combination of ingredients is used, so that the conidia of the entomopathogenic fungus remain stable, infective, and easy to apply. In this work, five different formulations were designed and produced, using the most pathogenic colonies towards *R. nu* larvae (LPSc

1082, LPSc 1156, LPSc 1226, LPSc 1086 and LPSc 1098) according to previous bioassay.

Mass production of conidia of each fungal colony was done in bioreactors (minicubes), where different substances (listed in Table 1) were incorporated into each formulation. A defoamer (polymer containing non-ionic emulsifiers) was utilized as an additive agent to reduce and hinder the formation of foam (Antifoam A®, Sigma-Aldrich). Polyoxyethylene sorbitol and polyethylene glycol (Tween 80®, Merck--Ultrapeg 4000, Oxiteno) (both non-ionic sufactants) were added to reduce conidia agglutination. Liquid pure vaseline (mineral oil) was utilized as a conidia carrier and protectant (Parafarm). A mix of methyl esters (vegetable oils, polimer of polyalkylene and silicone) was used as co-adjuvant in order to increase penetration and facilitate the cuticular absorption and also to promote a better spread and uniformity of the drops (Rizospray Extremo®, Rizobacter) (Rombach 1988; Mascarin *et al.* 2010; Lohse *et al.* 2014).

The oil-water combination was prepared by mixing the surfactant phase with the aqueous spore suspension, placing it in a beaker with a magnetic stirring rod and stirring for 60 min on a Velp brand magnetic stirrer, until a homogeneous combination was observed. Also, Triton X-100 was added, which was used as a non-ionic surfactant at a concentration of 1%. The colonies were grown in Erlenmeyer flasks and were posteriorly sown in mini-vats (10 l). The minicubes were set at 29°C, 200 RPM, 0.5 LPM, and 1.5 vvm. An Erlenmeyer flask containing defoamer was also connected to the minicubes in order to control the production of foam by the fungal strain. The colonies used in each of the combinations were grown for 6 days for each formulation. Once growth in minicubes was finished, after checking the purity with a microscope, they were stored in pyrex bottles for their subsequent combination with different additives. The conidia concentration in each formulation was adjusted to 1×10^8 conidia · ml^{-1}.

Laboratory test with fungal formulations

Larval mortality was determined using third instar larvae of *R. nu*. Each formulation was sprayed on individual larvae (300 µl) using a glass sprayer (discharge rate 0.10 ± 0.02 ml). Afterwards, larvae were placed in sterile containers (30 cm^3) with an artificial diet provided *ad libitum*. Each formulation was tested using three replicates (on different days) of ten individuals and a control group. The control larvae were sprayed in the same mode, but the fungal inoculum was not incorporated, only excipients of each formulation (Table 1) were present. The larvae were placed in a climatic chamber (25 ± 0.5°C, 75% RH, and 16/8 h L/D photoperiod) and the cumulative mortality was recorded for 10 days. Death due to mycosis was confirmed in the same fashion as in the pathogenicity assay. Using Infostat software, data were analyzed using an analysis of variance (ANOVA) to test for significant differences in mortality percentages between treatments, and then compared using a Tukey test (p = 0.05) (Di Rienzo *et al.* 2011).

Field test

The field trial was carried out using the two formulations that showed the highest mortality on *R. nu* larvae in previous laboratory tests. The field was located in the Pampeana biogeographic region in Buenos Aires province, Argentina (33°43‘53.2” S/ 60°30’32.1” W) which is the most important agricultural nucleus in the country and has constant attacks of lepidopteran pests. The trial consisted of two treatments and three controls. CoragenTM (rynaxypyr 20% [w/v], DuPont S.A., Argentina) is a chemical positive control that is widely used in Argentina to control *R. nu* and F4–F5 controls (containing all of the compounds of each formulation but without the fungal conidia). Six plots for each treatment were randomly selected. On each plot, four soybean furrows 50 cm long were delimited. Plants (R1 phenological stage) were selected by furrow. Twenty L3 larvae were placed on leaves of each plant using entomological tweezers and brushes to avoid causing any injury to the insects. The experimental design was a randomized block design, to reduce the experimental error that is mainly caused by the heterogeneity of the soil in the field, among other factors (Letourmy 1999; Ramírez-Godoy *et al.* 2018). Plants were covered with an anti-aphid net to prevent larvae from escaping (Fig. 1). The application of

Fig. 1. Anti-aphid net placed on soybean plants to prevent the larvae of *Rachiplusia nu* from moving to neighboring plants

Table 1. Different ingredients and concentrations used for the composition of each of the five fungal formulations tested

Formulated	Strain LPSc	Ingredients													
		H_2O [ml]	Defoamer [g · l^{-1}]	Tween 80 [g · l^{-1}]	Ultrapeg 4000 [g · l^{-1}]	Mineral oil [m · l^{-1}]	Glucose [g · l^{-1}]	NaCl [g · l^{-1}]	Medium L [m · l^{-1}]	Medium Cz [ml]	Medium G [ml]	Yeast extract [g · l^{-1}]	Buffer K_2HPO/ KH_2PO_4 [g · l^{-1}]	$CaCO_3$ [g · l^{-1}]	Rizospray Extremo [ml]
F1	1082	200	30.00	50.00	0.10	0.10	–	–	–	–	–	–	–	–	–
F2	1156	–	30.00	–	–	–	10.00	0.50	100	–	–	1.25	–	1.00	–
F3	1226	–	–	–	–	–	–	–	–	–	100	–	4.00/3.60	–	–
F4	1086	–	30.00	50.00	0.10	–	–	–	–	100	–	–	–	–	0.1
F5	1098	–	30.00	50.00	0.10	–	–	–	–	100	–	–	4.00/3.60	–	0.1

Medium L – yeast extract 25.00; melaza 5.00; defoamer 3 ml; $MgSO_4.7H_2O$ 0.30; $FeSO_4.7H_2O$ 0.05; $ZnSO_4.7H_2O$ 0.014 g · l^{-1}
Medium Cz – sucrose 30.0; $NaNO_3$ 2.0; magnesium glycerophosphate 0.5; KCl 0.5; $FeSO_4$ 0.01; K_2SO_4 0.35 g · l^{-1}
Medium G – glucose 50.00; starch 5.00; casein peptone 25.00; KCl 18.80; defoamer 3 ml; KH_2PO_4 2.00; $CaCl_2.2H_2O$ 0.40; $MgSO_4.7H_2O$ 0.30; $FeSO_4.7H_2O$ 0.05; $ZnSO_4.7H_2O$ 0.014; $MnSO_4.H_2O$ 0.016; $CoCl_2.6H_2O$ 0.037 g · l^{-1}

formulations was made with a backpack and a carbon dioxide (CO_2) bottle (Spraytec SRL). For field application Integrum® was used as an adjuvant, which was composed of a modified vegetable oil and six different surfactant molecules. This product was used to maximize the penetration of the tested compounds and reduce possible evaporation of the treatments. The formulation dose used was about 200 ml · ha^{-1}. Each plant was inspected daily to record larval mortality for a period of 10 days. The dead insects were removed and placed in sterile vials that were taken to the laboratory to confirm death due to mycosis as described in previous sections. The differences between treatments (chemical-fungi) in larval mortality were analyzed performing an analysis of variance (ANOVA) and *a posteriori* Tukey test ($p = 0.05$) using the software InfoStat version 2011 (Di Rienzo *et al.* 2011).

Results

Pathogenicity assays and laboratory tests with fungal formulations

Significant differences were observed in the pathogenicity of the 10 fungal colonies of *R. nu* L3 larvae ($df = 10$; $F = 2.53$; $p < 0.0045$). The *B. bassiana* strain LPSc 1098 caused the highest mortality (86.6 ± 8.4%), while *Metarhizium anisopliae* strain LPSc 907 showed the lowest mortality (56.6 ± 4.2%) (Fig. 2). On the other hand, no larvae died in the controls. As for laboratory tests with fungal formulations, significant differences were also observed ($df = 9$; $F = 9.14$; $p < 0.0001$). Formulation 4 showed the highest percentage of mortality (100%), followed by Formulation 5 (97 ± 1.3%) (Fig. 3). Formulation 1 caused the lowest mortality in *R. nu* larvae (83.3 ± 7.8%) (Fig. 3).

Field tests

Under field conditions, significant differences were observed ($df = 4$; $F = 8.09$; $p < 0.0001$) between the two fungal formulations for pathogenicity on *R. nu* larvae. Formulation 4 caused the highest mortality (82.4 ± 5.56%), while formulation 5 and chemical control caused a mortality of 61.8 ± 7.5% and 87.5 ± 3.2%, respectively (Fig. 4). Mortality in control treatments was below 20% in both cases (formulation 4 control, 5 ± 1.5% and formulation 5 control, 10 ± 2.3%) (Fig. 4).

Discussion

When selecting a method for mass production of entomopathogenic fungi, it is important to take into account not only the technological and economic feasibility, but also the mechanism of action, since infective structures and active metabolites must be

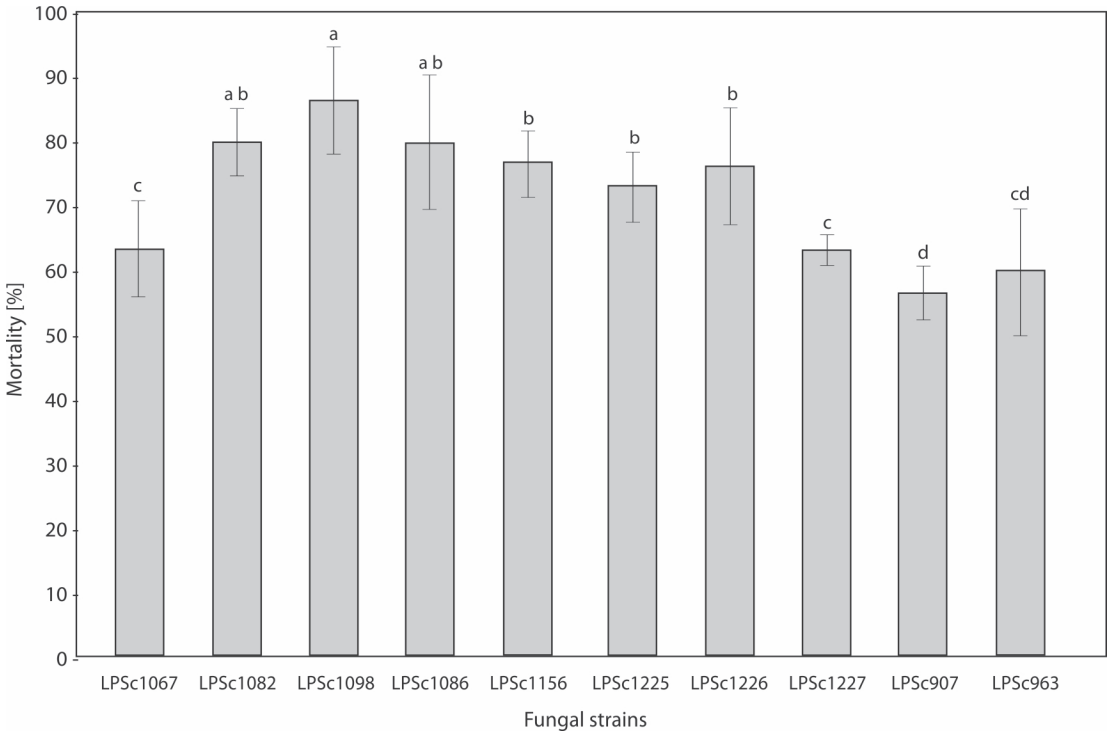

Fig. 2. Mean percent ± SD mortality of *Rachiplusia nu* larvae inoculated with different fungal colonies. The bars indicate standard errors and different letters indicate significant differences according to the Tukey test ($p \leq 0.05$)

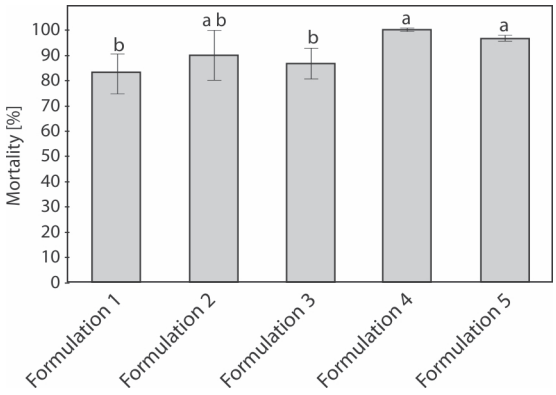

Fig. 3. Mean percent ± SD mortality in laboratory tests of *Rachiplusia nu* larvae inoculated with five fungal formulations. The bars indicate standard errors and different letters indicate significant differences according to the Tukey test ($p \leq 0.05$)

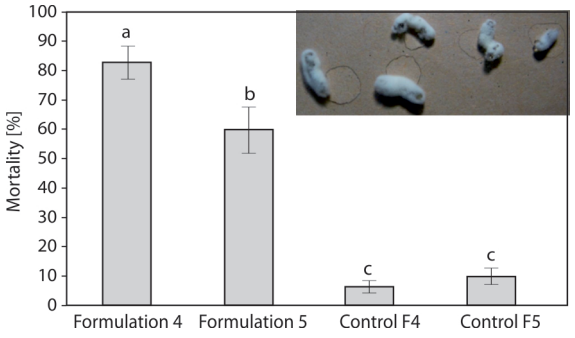

Fig. 4. Mean percent ± SD mortality in field tests of *Rachiplusia nu* larvae exposed to fungal formulations 4–5 and their respective controls. The bars show standard errors and different letters indicate significant differences according to the Tukey test ($p \leq 0.05$). Details of dead larvae by formulation of *Beauveria bassiana* are shown [in the upper right hand corner]

present in the final product, and show stability with its highest biological potential (Bansal *et al.* 1988; Ávila-Hernández *et al.* 2020). Solar radiation is one of the factors with the greatest damage potential to conidia and is responsible for the low persistence of mycoinsecticides in the field (Kaiser *et al.* 2020; Braga *et al.* 2001; Cagan and Svercel 2001). For this reason, UV protectants are always present in bioformulations in order to protect microbial agents in the field (Posadas *et al.* 2012). Likewise, the use of surfactants or adjuvants in foliar applications is a common practice to improve efficiency, provide a greater surface of coverage, and improve adhesion and penetration of the formulations. However, some studies have reported that certain oil-based surfactants can affect the viability of conidia and also the pathogenicity of entomopathogenic fungi (Pelizza *et al.* 2018). This study demonstrated compatibility with the utilized adjuvants, since conidial viability was greater than 95% in the different formulations.

The mortality percentages of *R. nu* larvae treated with different colonies of fungal entomopathogens ranged between 56.6 and 86.6%. González-Maldonado *et al.* (2015) showed similar results using *B. bassiana,* reporting mortalities of 50% in *Spodoptera frugiperda* larvae. Regarding the use of fungal formulations under laboratory conditions, formulation 4 with the strain (LPSc 1086) of *B. bassiana* exhibited the highest mortality percentage (100%), followed by formulation 5 with the strain (LPSc 1098) of *B. bassiana* (97%). Instead, Lohse *et al.* (2014) recorded average mortalities of 77% in larvae of the lepidoptera *Plutella xylostella* L. The utilization of adjuvants and other ingredients protects the microbial agents from the damage provoked by sunlight, improving their persistence in the environment (Reddy *et al.* 2008). In this study, the commercial adjuvant Rizospray Extremo® was incorporated in formulations F4 and F5 to protect conidia from environmental conditions.

The viability of conidia in fungal formulations used in this study is in accordance with those reported by Mascarin and Jaronsky (2015), with values greater than 95%. One of the main limitations of the adoption of bioformulations is the inconsistency of results regarding their effectivity, mainly under field conditions (Mishra *et al.* 2018). In this study, larval mortalities were 83% for formulation F4 and 60% for formulation F5 when applied in the field. These results agree with those obtained by El-Husseini (2019), where field application of conidia on sugar beet plants caused a decrease between 66.6 and 80% in the population of *Spodoptera exigua* (Hübner) larvae.

In this investigation, the effectiveness of mycoinsecticides was observed after one application. Certain studies report that a second application would increase the number of infective conidia, thus contributing to an increase in the biocontrol achieved by the formulations (Gatarayiha *et al.* 2011; Litwin *et al.* 2020; Kumar *et al.* 2021). Additional trials are needed to further confirm the pathogenicity of this bioformulation and also to determine how environmental variables can influence efficacy.

Although a greater number of tests under field conditions with the fungal formulation F4 are necessary, the results obtained in this work allow speculating that it is possible to use this formulation under field conditions to control *R. nu*, thus reducing the amount of chemical insecticides used to control this insect pest.

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