



ORIGINAL ARTICLE

Compatibility of chemical insecticides and entomopathogenic fungi for control of soybean defoliating pest, *Rachiplusia nu*

Sebastian A. Pelizza^{a,b,*}, Santiago Schalamuk^c, María R. Simón^d,
Sebastian A. Stenglein^e, Suani G. Pacheco-Marino^f, Ana C. Scorsetti^b

^a Centro de Estudios Parasitológicos y de Vectores (CEPAVE), CCT La Plata-CONICET-UNLP, La Plata, Buenos Aires, Argentina

^b Instituto de Botánica Carlos Spegazzini, FCNyM-UNLP, La Plata, Buenos Aires, Argentina

^c CEQUINOR, CCT-La Plata-CONICET-UNLP, La Plata, Buenos Aires, Argentina

^d Facultad de Ciencias Agrarias y Forestales, UNLP, La Plata, Buenos Aires, Argentina

^e Laboratorio de Biología Funcional y Biotecnología (BIOLAB)-CEBB-CONICET, Facultad de Agronomía de Azul, UNCPBA, Azul, Buenos Aires, Argentina

^f Instituto de Genética Veterinaria Ingeniero Fernando Noel Dulout (IGEVEV), CCT La Plata-CONICET, Argentina

Received 13 February 2017; accepted 17 June 2017

Available online 25 October 2017

KEYWORDS

Rachiplusia nu;
Entomopathogenic
fungi;
Pesticides;
Interactions;
Synergism

Abstract *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae) is one of the major lepidopteran pests defoliating soybeans (*Glycine max* Merrill) in Argentina. The combined use of chemical insecticides and entomopathogenic fungi is a promising pest-control option to minimize adverse chemical effects. In this work, we evaluated the interactions between five insecticides—two being considered biorational—and five fungal entomopathogenic strains under laboratory conditions in order to determine the possible usefulness of combinations of these agents against *R. nu*. The insecticides were tested for compatibility at four doses by *in vitro* bioassay and for the lethality of *R. nu* by inoculations at three doses. Fungal strains were applied at 1×10^8 , 1×10^6 , and 1×10^4 conidia/ml. The combinations of those insecticides with *Beauveria bassiana* (LPSc 1067, LPSc 1082, LPSc 1098), *Metarhizium anisopliae* (LPSc 907), and *Metarhizium robertsii* (LPSc 963) caused higher *R. nu*-larval mortalities than any of the individual agents alone. We observed significant differences in the *in vitro* conidial viability, vegetative growth, and conidia production of the five strains of entomopathogenic fungi exposed to different doses of the chemical insecticides. The combination gamma-cyhalothrin-LPSc-1067 caused the highest percent mortality of *R. nu* larvae, with synergism occurring between the two agents at 50% and 25% of the maximum field doses.

© 2017 Asociación Argentina de Microbiología. Published by Elsevier España, S.L.U. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author.

E-mail address: pelizza@cepave.edu.ar (S.A. Pelizza).

<https://doi.org/10.1016/j.ram.2017.06.002>

0325-7541/© 2017 Asociación Argentina de Microbiología. Published by Elsevier España, S.L.U. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

PALABRAS CLAVE

Rachiplusia nu;
Hongos
entomopatógenos;
Insecticidas;
Interacciones;
Sinergismo

Compatibilidad de insecticidas químicos y hongos entomopatógenos para el control de *Rachiplusia nu*, plaga defoliadora de la soja

Resumen *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae) es una de las principales plagas de lepidópteros defolios del cultivo de soja (*Glycine max* Merrill) en Argentina. El uso combinado de insecticidas químicos y hongos entomopatógenos es una opción de control de plagas prometedora para minimizar los efectos químicos adversos. En este trabajo se evaluaron las interacciones entre 5 insecticidas —2 de ellos considerados biorracionales— y 5 cepas fúngicas entomopatógenas en condiciones de laboratorio, para determinar la posible utilidad de combinaciones de estos agentes frente a *R. nu*. Se evaluó la compatibilidad de los insecticidas a 4 dosis mediante bioensayos *in vitro* y la letalidad de aquellos sobre *R. nu* mediante inoculaciones a 3 dosis. Las cepas fúngicas se evaluaron a concentraciones de 1×10^8 , 1×10^6 y 1×10^4 conidios/ml. Las combinaciones de estos insecticidas con *Beauveria bassiana* (LPSc 1067, LPSc 1082, LPSc 1098), *Metarhizium anisopliae* (LPSc 907) y *Metarhizium robertsii* (LPSc 963) causaron una mayor mortalidad de larvas de *R. nu* que cualquiera de los agentes individuales. Asimismo, se observaron diferencias significativas en la viabilidad de los conidios *in vitro*, el crecimiento vegetativo y la producción de conidios de las 5 cepas de hongos entomopatógenos expuestos a diferentes dosis de los insecticidas químicos. La combinación gamma-cialotrina-LPSc-1067 causó el mayor porcentaje de mortalidad de larvas de *R. nu*, con un efecto de sinergismo entre los 2 agentes al 50 y el 25% de las dosis de campo recomendadas por el fabricante.

© 2017 Asociación Argentina de Microbiología. Publicado por Elsevier España, S.L.U. Este es un artículo Open Access bajo la licencia CC BY-NC-ND (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Oilseeds and cereals are excellent field crops in Argentina, as they provide a major portion of the income and a high percentage of the food base for the country's population. The soybean—the main oilseed crop of Argentina—is also the most widespread, with a planted area of 20 million hectares and an annual production of 53 000 000 metric tons, equivalent to a value of 26 billion US dollars²². In the last 21 years, the total area planted with soybeans has increased 3.6-fold and the production 4.8-fold¹⁶. Argentina, for its part, is the third largest producer of soybeans in the world after the USA and Brazil and the first exporter of oil and soybean meal²². One of the main causes of decreasing in the average annual yields has been attributed to the attack of crops by insect pests. Agricultural crops are affected by a wide variety of insects, and in Argentina, at the year 2013, soybean crop losses caused by insect pests were computed as 10% of total annual production. The amount of chemical insecticide required for control pests was 30 million of kilograms valued in 260 US million dollars⁷.

Rachiplusia nu—a widespread species on the South-American continent—is particularly common in Argentina, Bolivia, Brazil, Chile, and Uruguay. The larval stage of *R. nu* can cause severe damage to crops including soybeans, sunflowers (*Helianthus annuus* L.), corn (*Zea mays* L.), wheat (*Triticum* sp L.), and alfalfa (*Medicago sativa* L.). In Argentina, this insect represents major pests of soybean crops. The pest infestations usually start from December and reach maximum levels in January and February¹⁶. The early instars larvae of *R. nu* feed on leaf parenchyma without damaging the leaf veins, reaching their highest voracity during their fifth larval instar, eating during their larval period,

between 100 and 110 cm² of soybean leaves. This decrease in leaf area, results in lower sunlight interception, lower photosynthetic capacity, loss of material stored in leaf and shortening the period of grain filling³¹. To date, farmers use broad-spectrum insecticides to combat this pest, but the indiscriminate use of these chemical agents is problematic and undesirable because of their poor selectivity, residual toxicity in the environment, bioaccumulation, and high frequency of resistance formation in insects¹³. According to the Food and Agriculture Organization of the United States⁹, a comprehensive public policy is essential for plant health. Five major aspects of today's social and economic reality in Argentina are¹: the decisive role in plant health requirements for international trade²; the urgency of the need for the quality control of food safety in the diet, especially within the context of the exportation of foods³; the need for significant improvements in the safety of rural agricultural workers⁴; the environmental quality of the rural and small urban settlements; and finally⁵ the conservation of natural resources, especially water.

For these reasons, the biologic control of insect pests through the use of pathogens, parasites, and predators has received particular attention in recent years. Among these microorganisms, the role of entomopathogenic fungi as prominent biologic-control agents has been the subject of intense research for over 100 years³⁴. The fungal species *Beauveria bassiana* (Bals.-Vuill.) Vuillemin and *Metarhizium anisopliae* (Metschn.) Sorokin are the most commonly used for the biologic control of the insect pests of agricultural crops^{18,25}. Moreover, less used in biological control, but not least, is the case of insect-pathogenic fungus, *Metarhizium robertsii* J.F. Bisch., Rehner & Humber. It is a common inhabitant of soils worldwide³ and has been studied and used

as an insect pathogen for biocontrol^{15,20}. A disadvantage, however, of using entomopathogenic fungi in pest-control programs for field crops is the excessive time required to kill the target insect²⁰. For this reason, the combined use of entomopathogenic fungi plus sublethal doses of chemical insecticides has been found to be a promising alternative for reducing the amount of chemicals used for pest control, thus protecting the health of the environment and the agricultural workers⁵.

In this study, we evaluated, under laboratory conditions, the interactions between five insecticides, two being considered bio rational, and five strains of entomopathogenic fungi in order to determine the possible usefulness of combinations of these agents against the soybean-defoliating lepidopteran pest *R. nu*.

Materials and methods

Insect rearing

The *R. nu* individuals used in the toxicity tests were obtained from a colony (provided by the company Agldea [Agricultural Innovation Applied Research]) without history of insecticide exposure. The larvae were kept on a semi synthetic diet¹⁴ in a controlled chamber at $25 \pm 0.5^\circ\text{C}$, a 75% relative humidity, and a light/dark photoperiod of 16/8 h, during the trial time.

Fungal strains

The entomopathogenic fungi used in this study were obtained from Spegazzini Institute culture collection, LPSc 1067 (GenBank accession number KF500409), LPSc 1082 (GenBank accession number KJ7722495) and LPSc 1098 of *B. bassiana*, LPSc 907 (GenBank accession number KJ772494) of *M. anisopliae* and LPSc 963 of *M. robertsii* J.F. Bisch., Rehner & Humber. All isolates were preserved by freeze drying (lyophilization) technique. The provisional species identification (LPSc 1098 and LPSc 963) by morphology was corroborated by genetic analysis after extracting the DNA of the monospore cultures according to Stenglein and Balatti³³. Polymerase-chain reactions (PCRs) were carried out in an XP thermal cycler (Bioer Technology Co, Hangzhou, China) to amplify and sequence the internal-transcribed-spacer rDNA region of *B. bassiana* through the use of the primer pairs ITS5/ITS4³⁰ and the translation-elongation factor 1-alpha (the elongation-factor-intron sequence) of *M. anisopliae* by means of the primer pairs EF1T/EF2T⁴. Fragment similarities to previously published sequence data were examined by BLASTn¹ in the web page of the National Center for Biotechnology Information. The sequences generated in this study were submitted to GenBank (accession numbers KT163259 for *B. bassiana* LPSc 1098; and KT163258 for *M. robertsii* LPSc 963).

BLAST searches of the LPSc 1098 showed 100% of similarity (e-value 0.0) with *B. bassiana* (e.g., accession numbers KU726564, KU158472, KT443982) and for LPSc 963 sequence showed 100% of similarity (e-value=0.0) with several sequences of *M. robertsii* (e.g., accession numbers KT862515, KR70649, KP027978).

The choice of these fungal strains was based on their laboratory efficacy against pest grasshopper and locust species from Argentina^{27,28}. The conidia of the fungal

strains obtained from cultures on potato-dextrose-agar (PDA) medium (Britania S.A., Buenos Aires, Argentina) after incubation for 10 days at 25°C in the dark were harvested with disposable cell scrapers (FisherbrandTM) and placed in test tubes containing 0.01% (v/v) polyoxyethylene sorbitan monolaurate (Tween 80TM; Merck). The resulting suspensions were vortexed for 2 min, filtered through four layers of sterile muslin, and adjusted to 1×10^8 , 1×10^6 , and 1×10^4 conidia/ml after cell counting in a Neubauer hemocytometer. The viability of the conidia from each isolate and the concentrations used in the tests were determined after 24 h as described by Goettel and Inglis¹². This germination test was repeated for each stock suspension to maintain the constancy of the viability assessments. In all instances, the average viability of the conidia was over 95%.

Insecticides

The synthetic chemical pesticides Archer PlusTM (gamma-cyhalothrin 15% [w/v], Cheminova SA, Argentina), LambdaTM (lambda-cyhalothrin 25% [w/v], Handelsgesellschaft Detlef Von Appen, Argentina), and CoragenTM (rynaxypyr 20% [w/v], DuPont S.A., Argentina), along with two biorational chemical insecticides, MatchTM (luphenuron 5% [w/v], Sygenta S.A., Argentina) and IntrepidTM (methoxyfenozide 24% [w/v], Dow AgroSciences S.A., Argentina) were used in these experiments.

The insecticides were tested for fungal compatibility at four doses by *in vitro* bioassay: twice the average concentration recommended for field application on soybean (200%), the average recommended (100%), 50% of the average, and finally 25%; to give levels of 104, 52, 26, and 13 ppm (mg active ingredient/l [a.i./l]) for gamma-cyhalothrin; 174, 87, 44, and 22 ppm (mg a.i./l) for lambda-cyhalothrin; 120, 60, 30, and 15 ppm (mg a.i./l) for rynaxypyr; 200, 100, 50, and 25 ppm (mg a.i./l) for luphenuron; and 288, 144, 72, and 36 ppm (mg a.i./l) for methoxyfenozide⁷, and at three doses in the experimental inoculations against *R. nu*: the average recommended for field application on soybean (100%), 50% of the average, and finally 25%; to give levels.

In vitro compatibility of fungal isolates with chemical insecticides

Assessment of germination

The conidial viability of was measured as described by Goettel and Inglis¹². A suspension of 1×10^6 conidia/ml in Tween 80TM was vortexed for 2 min, then filtered through four layers of sterile muslin. Of this suspension, 1 ml was combined with 1 ml of each of the four doses of insecticides indicated in the previous section and then incubated for 1 h at room temperature. Next, 800 μl of sterile PDA culture medium was layered onto each of a series of autoclaved microscope slides at a thickness of about 2 mm and the slides transferred to Petri dishes containing a filter-paper disk. To each of those dishes, 0.5 ml of one of the fungus-insecticide admixtures was spread over the agar-coated slide. The dishes were then incubated at 25°C for a single 12/12-h photoperiod and the percent germinated conidia quantified 24 h later. A control without the addition of chemical insecticide was made for each fungal isolate. The conidia were considered to have germinated once the germ tube had reached

half a conidium's length. Each assessment was made in triplicate per treatment and 300 conidia counted under each experimental condition. The data were analyzed through the use of the generalized linear model with the software GenStat Twelfth Edition¹¹, with a binomial error distribution and logit-link function. The means were compared according to the least significant difference (LSD; $p \leq 0.05$).

Assessment of vegetative growth and conidia production

The chemical insecticides were incorporated into the PDA medium supplemented with streptomycin (0.5 g/l), at $45 \pm 5^\circ\text{C}$, and poured into Petri dishes at room temperature as described by Neves et al.²³ After medium solidification, the fungi were inoculated with a sterile punch of 1 cm^2 in the center of the Petri dish, (at five dishes per treatment). The dishes were then incubated at $25 \pm 1^\circ\text{C}$ for a 12/12-h photoperiod. The colony areas were finally measured on vegetable paper with a planimeter at 7 days for *B. bassiana* (LPSc 1067, LPSc 1082, LPSc 1098) and 10 days for *M. anisopliae* (LPSc 907) and *M. robertsii* (LPSc 963) after the inoculation. Once the areas of the colonies were drawn, 10 disks (2 cm^2) from the center of the colony in each treatment were collected for the quantification of conidia production. Each disk was placed in a glass tube and the conidia suspended in 10 ml of 0.01% (v/v) Tween 80TM and counted in a Neubauer chamber. The data were analyzed according to the generalized linear model with the software GenStat Twelfth Edition¹¹, with a logarithmic Poisson-distribution function for conidia production and a normal distribution function for vegetative growth. The means were compared by LSD ($p \leq 0.05$).

Experimental inoculations

Experimental inoculations were conducted on *R. nu* third-instar larvae in order to determine the percent mortality at each dose of the insecticide (100, 50 and 25% the average field recommended) and at different concentration of fungal strains (1×10^8 , 1×10^6 , and 1×10^4 conidia/ml). Each larva was sprayed with 300 μl of each treatment through the use of a glass fine mist sprayer (discharge rate $0.10 \pm 0.02\text{ ml}$). The larvae were inoculated individually and then placed in sterile containers (30 cm^3) with an artificial food available *ad libitum*. Three replicates and one control of 10 individuals each was used with each of the treatments. The larval controls were sprayed in the same mode, but with the vehicle 0.01% [v/v] Tween 80TM alone. Both the treated and the control insects were maintained at $25 \pm 0.5^\circ\text{C}$, a 75% relative humidity, and a light/dark photoperiod of 16/8 h. The cumulative mortality was recorded daily for 10 days. The dead larvae with no external mycelia were surface-sterilized by successive immersions in 70% ethanol (10–15 s), 0.5% (w/v) sodium hypochlorite solution (1 min), and sterile distilled water (1 min, two consecutive dips) according to Lacey and Brooks¹⁹. Next, the specimens were placed in sterile Petri dishes (60 mm diameter) with filter-paper disks periodically moistened with sterile distilled water and the dishes incubated at 25°C in the dark for 3–5 days for fungal development. Mycosis was confirmed by microscopical examination of the dead larvae. When the mortality in the treated larvae at different concentrations of the

five fungal strains and different dose of chemical compounds was 50% or higher, the median survival time (MST) in days were calculated based on the Kaplan–Meier survival-distribution function³⁵. Pairwise comparisons between the survival curves were made by the Wilcoxon test. Moreover, when the mortality was 50% or higher in the larvae treated with the different chemical compounds alone, the following lethal and sublethal-toxicity parameters were calculated (DEBtox v. 2.0.1 program)^{17,29} the median lethal concentration (LC_{50}), the concentration lethal to 10% of the exposed individuals (LC_{10}), and the no-effect concentration (NEC). Significant differences between the percent mortalities after a 10-day exposure to the different treatments were analyzed according to the generalized linear model with a binomial distribution and logit-link function. The means were compared by the LSD test ($p \leq 0.05$).

Interaction between entomopathogenic fungi and chemical insecticides against *R. nu*

The interaction of *B. bassiana* (LPSc 1067, LPSc 1082, LPSc 1098), *M. anisopliae* (LPSc 907) and *M. robertsii* (LPSc 963) with the insecticides gamma-cyhalothrin, lambda-cyhalothrin, rynaxypyr, lufenuron, and methoxyfenozide against *R. nu* third-instar larvae was investigated in the following manner. Of each fungal suspension and each insecticide solution, 5 ml were combined, to give a final volume of 10 ml (these were adjusted to reach the abovementioned concentration for each fungal strain and chemical insecticide evaluated). Suspensions were homogenized by vortexing for 2 min. The experimental design and inoculation techniques were performed as indicated in the previous section. The cumulative mortality was recorded daily for 10 days and mycosis confirmed by microscopical examination of the dead larvae. When the mortality reached 50% or higher, the toxicologic effect of gamma-cyhalothrin, lambda-cyhalothrin, rynaxypyr, lufenuron, and methoxyfenozide on those *R. nu* third-instar larvae were calculated. The LC_{10} – LC_{50} and NEC values were expressed as ppm, and the MST in days, both parameters along with the respective standard deviations (SDs). Significant differences between the percent mortalities after a 10-day exposure to the different pesticide combinations were analyzed according to the generalized linear model with a binomial distribution and logit-link function. The means were compared by the LSD test ($p \leq 0.05$).

The determination of additive, synergistic, or antagonistic interaction was based on a binomial test involving a comparison of the expected and observed percentage mortalities as described by Nishimatsu and Jackson²⁴. The expected mortality at a set concentration of insecticide and fungal strain was based on the formula $P_e = P_o + (1 - P_o)(P_1) + (1 - P_o)(1 - P_1)(P_2)$, where P_e is the expected mortality on combination of the 2 insecticidal agents, P_o the natural (control) mortality, P_1 the mortality after treatment with the insecticide alone, and P_2 the mortality, after treatment with the fungal strain alone. Additivity was indicated if $\chi^2 < 3.84$. Antagonism was indicated if $\chi^2 > 3.84$ and $P_c < P_e$, where P_c is the observed mortality of the insecticide and fungal-strain combination. Synergism was indicated if $\chi^2 > 3.84$ and $P_c > P_e$.

Table 1 Results from the generalized linear model on the effects of the chemical insecticides on conidial viability, vegetative growth, and conidia production in the fungal strains

Parameter	Source	DF	Chi-Square	Pr > ChiSq
Conidial viability	Insecticides	20	27.51	<0.1
	Strains	4	173.71	<0.1
	Insecticides*Strains	80	1.23	<0.08
Vegetative growth	Insecticides	20	51.20	<0.001
	Strains	4	84.27	<0.001
	Insecticides*Strains	80	14.32	<0.001
Conidia production	Insecticides	20	284 945 722.60	<0.001
	Strains	4	355 937 902.12	<0.001
	Insecticides*Strains	80	9 316 535.59	<0.001

Results

We observed significant differences in the *in vitro* conidial-viability test among the different insecticides and among the five fungal strains alone as well as in the interaction between the insecticides and the strains (hereafter, referred to as *insecticides*strains*) as defined by a $p \leq 0.083$ (Table 1). Reductions occurred in the percent conidial germination in all the fungal isolates with increased doses of the chemical insecticides: thus, a greater decrease occurred when the dose of insecticide was twice that recommended for field application by the manufacturer (Table 2). The insecticides that less affected the conidial germination of the different isolates were gamma- and lambda-cyhalothrin (Table 2). Significant differences likewise obtained in the vegetative growth of the fungi among the different fungal strains either alone or exposed to the five chemical insecticides (Table 1). The most significant reduction was observed in the colony area for the *B. bassiana* strains LPSc 1082, LPSc 1098 and *M. anisopliae* strain LPSc 907, when grown on PDA medium at a dose of 200 ppm lufenuron. In contrast, the vegetative growth of the strain LPSc 1067 was not affected on the same medium supplemented with gamma-cyhalothrin, lambda-cyhalothrin, and rynaxypyr; even at the highest doses used of 104, 174, and 120 ppm, respectively; while the colony area of the fungal strain LPSc 907 was likewise unchanged by gamma-cyhalothrin (Table 2). In a similar manner, the vegetative growth of LPSc 963 remained constant in the presence of methoxyfenozide and that of LPSc 1082 and LPSc 1098 was undisturbed with lambda-cyhalothrin in the medium, also at the highest doses used. In general, the chemical insecticides gamma-cyhalothrin, lambda-cyhalothrin, and rynaxypyr were the three that least affected the vegetative growth of most of the fungal isolates studied, though gamma-cyhalothrin and lambda-cyhalothrin did produce a significant inhibition of the vegetative growth of LPSc 963 (Table 2).

Finally, we found significant differences in the conidia production from all five strains of entomopathogenic fungi when maintained in association with different doses of chemical insecticides (Table 1). The reduction in conidia production was more pronounced with lufenuron and methoxyfenozide at the higher doses of 200 ppm and 288 ppm, respectively (Table 2). *M. robertsii* strain LPSc 963 was the most greatly affected by the action of

these chemical insecticides, with conidia productions that ranged from $2.6 \times 10^7 \pm 2.2 \times 10^3$ conidia/ml in the control to $3.4 \times 10^5 \pm 2.6 \times 10^2$ conidia/ml when combined with 200 ppm lufenuron and $3.1 \times 10^5 \pm 2.5 \times 10^2$ conidia/ml when exposed to 288 ppm of methoxyfenozide (Table 2). Insecticides that affected conidia production to a lesser extent even at higher doses were gamma-cyhalothrin and lambda-cyhalothrin. *B. bassiana* strain LPSc 1067 in particular was the least affected by these latter two insecticides, with conidia-production values ranging from $8.3 \times 10^7 \pm 4^3$ conidia/ml in the control to $8.4 \times 10^6 \pm 1.2 \times 10^3$ conidia/ml in combination with 104 ppm of gamma-cyhalothrin and $4.6 \times 10^6 \pm 9.6 \times 10^2$ conidia/ml when exposed to 174 ppm of lambda-cyhalothrin (Table 2).

A comparison of the percent mortalities of third-instar larvae of *R. nu* registered after a 10-day exposure to different combinations of the five insecticides and the five fungal strains revealed significant differences. The interaction insecticides*strains was significant at doses of 50% of the maximum field concentration, but not at 100% or 25% (Table 3). In addition, the percent mortality obtained after exposure of the larvae to the insecticides or to the fungal strains alone was 100%, except for *B. bassiana* LPSc 1098 and *M. robertsii* LPSc 963, where the percent mortalities were $80 \pm 0.78\%$ and $70 \pm 0.67\%$, respectively (Fig. 1A).

Moreover, exposure of *R. nu* to strains LPSc 1067, LPSc 1082, LPSc 1098, LPSc 907, or LPSc 963 in combination with any one of the insecticides at the maximum field concentrations produced 100% mortality in the larvae in all instances (Fig. 1A). At those maximum field concentrations of both entomopathogenic agents, however, we observed an additive effect with all those combinations (Table 4). At 50% of the maximum concentrations of both the chemical insecticides and the fungal isolates, we also observed a greater percent of mortality of the *R. nu* larvae after exposure to the two agents in combination than after treatments with those compounds or the fungi alone—except for the combinations lambda-cyhalothrin–LPSc-1098 ($30 \pm 0.48\%$), whose lethality was lower than that of the insecticide alone ($40 \pm 0.23\%$), or all combinations of the *M. robertsii* strain LPSc 963, where the percent mortality in combination with any of the chemical insecticides was lower than that caused by those insecticides or the isolate alone (Fig. 1B). In contrast, at this 50% maximum concentration

Table 2 Conidial viability, vegetative growth and conidia production of strains *Beauveria bassiana* LPSc 1067, LPSc 1082, LPSc 1098; *Metarhizium anisopliae* LPSc 907; and *Metarhizium robertsii* LPSc 963; both alone and in combination at twice the average concentration of the chemical agents recommended for application in the field (200%), at the average concentration (100%), and at concentrations reduced to 50% and 25% of the average

Insecticides	Doses (ppm)	Fungal strains*								
		LPSc 1067			LPSc 907			LPSc 1082		
		V	VG	CP	V	VG	CP	V	VG	CP
Gamma-cyhalothrin	0	98 ± 2.4a	9 ± 0a	8.3 ⁷ ± 4 ³ a	97 ± 2.1a	9 ± 0a	8.9 ⁷ ± 4.2 ³ a	94 ± 2.4a	9 ± 0a	1.6 ⁷ ± 1.7 ³ a
	13	97 ± 1.8a	9 ± 0a	8 ⁷ ± 4 ³ a	97 ± 1.3a	9 ± 0a	8.8 ⁷ ± 4.2 ³ a	94 ± 2.1a	9 ± 0a	1.5 ⁷ ± 1.7 ³ a
	26	95 ± 2.2bc	9 ± 0a	3.1 ⁷ ± 2.5 ³ b	97 ± 1.9a	9 ± 0a	1.4 ⁷ ± 1.6 ³ b	94 ± 2.5a	9 ± 0a	3 ⁶ ± 7 ² b
	52	96 ± 1.4ab	9 ± 0a	1.4 ⁷ ± 1.7 ³ c	95 ± 2.5a	9 ± 0a	6.4 ⁶ ± 1.1 ³ c	92 ± 2.7a	9 ± 0a	3.4 ⁶ ± 8 ² b
	104	93 ± 1.2c	9 ± 0a	8.3 ⁶ ± 1.2 ³ d	92 ± 2.2b	8.2 ± 0.8a	5.3 ⁶ ± 1 ³ c	90 ± 3.2b	9 ± 0a	3.4 ⁶ ± 8 ² b
Lambda-cyhalothrin	0	98 ± 2.4a	9 ± 0a	8.3 ⁷ ± 4 ³ a	97 ± 2.1a	9 ± 0a	8.9 ⁷ ± 4.2 ³ a	94 ± 2.4a	9 ± 0a	1.6 ⁷ ± 1.7 ³ a
	22	98 ± 2.4a	9 ± 0a	5.1 ⁷ ± 3.2 ³ b	97 ± 2.8a	9 ± 0a	2.1 ⁷ ± 2 ³ b	91 ± 3.4a	9 ± 0a	1.4 ⁷ ± 1.6 ³ a
	44	97 ± 1.7a	9 ± 0a	1.5 ⁷ ± 1.7 ³ c	96 ± 1.7a	9 ± 0a	6.3 ⁶ ± 1.1 ³ c	91 ± 2.1a	9 ± 0a	9.2 ⁶ ± 1.3 ³ b
	87	96 ± 2.3ab	9 ± 0a	9.1 ⁶ ± 1.3 ³ d	94 ± 1.4a	9 ± 0a	4.2 ⁶ ± 9.2 ² c	92 ± 2.8a	9 ± 0a	5 ⁶ ± 1 ³ c
	174	92 ± 4.4b	9 ± 0a	4.6 ⁶ ± 9.6 ² e	89 ± 3.8b	5.8 ± 1.6b	3.6 ⁶ ± 8.5 ² c	91 ± 2.5a	9 ± 0a	3.4 ⁶ ± 8.2 ³ c
Rynaxypyr	0	98 ± 2.4a	9 ± 0a	8.3 ⁷ ± 4 ³ a	97 ± 2.1a	9 ± 0a	8.9 ⁷ ± 4.2 ³ a	94 ± 2.4a	9 ± 0a	1.6 ⁷ ± 1.7 ³ a
	15	97 ± 1.5a	9 ± 0a	2 ⁷ ± 2 ³ b	97 ± 3.6a	9 ± 0a	5.9 ⁶ ± 1 ³ b	93 ± 3.2ab	9 ± 0a	8.8 ⁶ ± 1.3 ³ b
	30	96 ± 2.2a	9 ± 0a	1.5 ⁷ ± 1.7 ³ b	95 ± 3.4a	9 ± 0a	5.6 ⁶ ± 1 ³ b	92 ± 2.8ab	9 ± 0a	8.4 ⁶ ± 1.2 ³ bc
	60	96 ± 1.8a	9 ± 0a	5.9 ⁶ ± 1 ³ c	94 ± 2.2ab	8.8 ± 1.2a	3.6 ⁶ ± 8.5 ² b	90 ± 1.5ab	8 ± 1.1a	4.5 ⁶ ± 9 ² cd
	120	92 ± 1.3b	9 ± 0a	4.3 ⁶ ± 9.3 ² c	90 ± 2.8b	6.3 ± 0.8b	3.4 ⁶ ± 8.3 ² b	89 ± 2.2b	6 ± 1.4b	1.3 ⁶ ± 5.1 ² d
Luphenuron	0	98 ± 2.4a	9 ± 0a	8.3 ⁷ ± 4 ³ a	97 ± 2.1a	9 ± 0a	8.9 ⁷ ± 4.2 ³ a	94 ± 2.4a	9 ± 0a	1.6 ⁷ ± 1.7 ³ a
	25	95 ± 2.3b	9 ± 0a	1.5 ⁷ ± 1.7 ³ b	93 ± 3.3b	5.4 ± 1.7b	6.6 ⁶ ± 1.1 ³ b	91 ± 2.7ab	9 ± 0a	8.4 ⁶ ± 1.2 ³ b
	50	93 ± 2.6bc	7.6 ± 1.1b	5.2 ⁶ ± 1 ³ c	93 ± 2.9b	2.7 ± 1.4c	4.7 ⁶ ± 9.7 ² b	90 ± 3.2b	8.6 ± 1.7a	7.9 ⁶ ± 1.2 ³ b
	100	90 ± 2.2cd	4.6 ± 0.8c	2.8 ⁶ ± 7.5 ² c	90 ± 2.3bc	2.4 ± 0.5cd	3.9 ⁶ ± 8.8 ² b	89 ± 2.3b	3.1 ± 0.6b	3.8 ⁶ ± 8.7 ² c
	200	87 ± 1.8d	1.8 ± 0.6d	2.4 ⁶ ± 7 ² c	87 ± 1.7c	1.2 ± 0.2d	3.3 ⁶ ± 8.1 ² b	85 ± 2.8c	1 ± 0.3c	2.5 ⁶ ± 7.2 ² c
Methoxyfenocide	0	98 ± 2.4a	9 ± 0a	8.3 ⁷ ± 4 ³ a	97 ± 2.1a	9 ± 0a	8.9 ⁷ ± 4.2 ³ a	94 ± 2.4a	9 ± 0a	1.6 ⁷ ± 1.7 ³ a
	36	95 ± 2.6b	8.7 ± 2.1a	1.2 ⁷ ± 1.5 ³ b	93 ± 3.1b	6.6 ± 1.9b	8 ⁵ ± 4 ² b	90 ± 2.1b	9 ± 0a	6.1 ⁶ ± 1.1 ³ b
	72	91 ± 1.5c	7 ± 1.1b	4.9 ⁶ ± 9.9 ² c	92 ± 3.6b	5.3 ± 2.1bc	5.9 ⁵ ± 3.4 ² b	90 ± 2.8b	8.2 ± 2.1a	4.2 ⁶ ± 9.2 ² bc
	144	89 ± 1.8c	6.7 ± 0.9b	2.7 ⁶ ± 7.4 ² cd	89 ± 2.8b	5.1 ± 2.9c	4.8 ⁵ ± 3.1 ² b	88 ± 3.1b	5.3 ± 2.2b	2.5 ⁶ ± 7 ² cd
	288	82 ± 2.3d	6.7 ± 1.2b	9.6 ⁶ ± 4.3 ² d	89 ± 2.2b	3.1 ± 2.7d	3.7 ⁵ ± 2.7 ² b	88 ± 3.4b	4.5 ± 1b	3.3 ⁵ ± 5 ² d

Table 2 (Continued)

Insecticides	Doses (ppm)	Fungal strains*					
		LPSc 1098			LPSc 963		
		V	VG	CP	V	VG	CP
Gamma-cyhalothrin	0	95 ± 3.6a	9 ± 0a	1.6 ⁷ ± 1.8 ³ a	92 ± 3.1a	9 ± 0a	2.6 ⁷ ± 2.2 ³ a
	13	93 ± 3.1ab	7 ± 2.4b	1.5 ⁷ ± 1.7 ³ a	90 ± 3.8a	3.4 ± 0.8b	2.5 ⁷ ± 2.2 ³ a
	26	93 ± 3.4ab	7 ± 2.5b	9.3 ⁶ ± 1.3 ³ b	92 ± 3.3a	2.7 ± 0.3b	1.1 ⁷ ± 1.5 ³ b
	52	91 ± 2.9b	7 ± 2.2b	8.8 ⁶ ± 1.3 ³ b	90 ± 3.6a	2.7 ± 0.6b	5.7 ⁶ ± 1 ³ c
	104	85 ± 3.8c	6.7 ± 2.3b	3.8 ⁶ ± 8.7 ² c	79 ± 4.6b	2.2 ± 1.1b	4.2 ⁶ ± 9.2 ² c
Lambda-cyhalothrin	0	95 ± 3.6a	9 ± 0a	1.6 ⁷ ± 1.8 ³ a	92 ± 3.1a	9 ± 0a	2.6 ⁷ ± 2.2 ³ a
	22	91 ± 3.8ab	9 ± 0a	8 ⁶ ± 1.2 ³ b	90 ± 2.5ab	2.4 ± 0.4b	7.4 ⁶ ± 1.2 ³ b
	44	90 ± 3.5bc	9 ± 0a	6.9 ⁶ ± 1.1 ³ bc	87 ± 1.8b	2.3 ± 0.9b	4.8 ⁶ ± 9.8 ² bc
	87	86 ± 3.4cd	9 ± 0a	4.5 ⁶ ± 9.5 ² bc	80 ± 1.1c	2 ± 0.7b	4.3 ⁶ ± 9.3 ² bc
	174	83 ± 3.3d	9 ± 0a	3.7 ⁶ ± 8.6 ² c	72 ± 1.1d	1.4 ± 0.4b	2.7 ⁶ ± 7.3 ² c
Rynaxypyr	0	95 ± 3.6a	9 ± 0a	1.6 ⁷ ± 1.8 ³ a	92 ± 3.1a	9 ± 0a	2.6 ⁷ ± 2.2 ³ a
	15	91 ± 3.3b	8.8 ± 2.3a	5.1 ⁶ ± 1 ³ b	87 ± 3.5b	7.6 ± 1.8ab	5.2 ⁶ ± 1 ³ b
	30	88 ± 3.2bc	8.4 ± 2.5a	4.5 ⁶ ± 9.5 ² b	83 ± 3.1b	7.2 ± 2.4bc	4.6 ⁶ ± 9.6 ² bc
	60	87.3 ± 3.5bc	7.6 ± 2.2ab	4.3 ⁶ ± 9.3 ² b	77 ± 2.8c	6 ± 2c	3.9 ⁶ ± 8.8 ² bc
	120	85 ± 4.1c	6.2 ± 2.3b	2.5 ⁶ ± 7.2 ² b	75 ± 3.3c	4.2 ± 2.2d	2.1 ⁶ ± 6.5 ² c
Luphenuron	0	95 ± 3.6a	9 ± 0a	1.6 ⁷ ± 1.8 ³ a	92 ± 3.1a	9 ± 0a	2.6 ⁷ ± 2.2 ³ a
	25	91 ± 4.8ab	7.5 ± 1.2b	4.5 ⁶ ± 9.5 ² b	83 ± 3.6b	8.4 ± 1.6ab	1.8 ⁶ ± 6 ² b
	50	90 ± 5.1bc	6.9 ± 1.1b	3.4 ⁶ ± 8.3 ² b	77 ± 4.8c	8.4 ± 1.4ab	6.9 ⁵ ± 3.7 ² b
	100	87 ± 4.4c	1.3 ± 0.8c	5.1 ⁶ ± 6.8 ² b	73 ± 4.1cd	7.1 ± 1.2b	4.3 ⁵ ± 2.9 ² b
	200	78 ± 4.7d	1.2 ± 0.1c	4.5 ⁶ ± 4.5 ² b	71 ± 4.5d	2.8 ± 0.6c	3.4 ⁵ ± 2.6 ² b
Methoxyfenocide	0	95 ± 3.6a	9 ± 0a	1.6 ⁷ ± 1.8 ³ a	92 ± 3.1a	9 ± 0a	2.6 ⁷ ± 2.2 ³ a
	36	89 ± 3.7b	8.4 ± 1.7ab	4.3 ⁶ ± 9.2 ² b	83 ± 3.8b	9 ± 0a	8.1 ⁵ ± 4 ² b
	72	87 ± 5.1bc	7.4 ± 1.1b	2.5 ⁶ ± 7.1 ² bc	75 ± 3.9c	9 ± 0a	4.5 ⁵ ± 3 ² b
	144	84 ± 5.5cd	5.3 ± 1.4c	1.5 ⁶ ± 5.5 ² bc	69 ± 4.7d	9 ± 0a	4.3 ⁵ ± 2.9 ² b
	288	80 ± 4.4d	5.5 ± 0.8c	3.3 ⁵ ± 2.5 ² c	68 ± 4.5d	8.4 ± 1.1a	3.1 ⁵ ± 2.5 ² b

CP = $a \times 10^b$ conidia/ml ± SD; V = mean percent ± SD; VG = cm² ± SD.

* Mean values followed by the same letters within the same fungal strain are not significantly different according to LSD ($p \leq 0.05$). Where large mean values and similarly large standard deviations occur—in the interest of space, we have resorted to using a shorthand notation for expressing those means and the standard deviations as well: Where a base 10 raised to a power would normally be written we have eliminated the base and simply placed the power to which it is raised immediately after the associated number so that what would otherwise, for example, be $a \times 10^b$ is written here merely as a^b.

Table 3 Results of the generalized linear model on the percent mortalities of third instar *R. nu* larvae after a 10-day exposure at different concentrations of chemical pesticides alone or in combination with fungal strains

Dose	100%			50%			25%		
	DF	Chi-Square	Pr > ChiSq	DF	Chi-Square	Pr > ChiSq	DF	Chi-Square	Pr > ChiSq
Insecticides	5	14.40	<0.001	5	19.34	<0.001	5	12.76	<0.001
Strains	5	7.61	<0.001	5	32.03	<0.001	5	14.05	<0.001
Insecticides*Strains	24	0.00	1.00	24	2.05	0.002	24	1.26	<0.175

of the two entomopathogenic agents we observed synergism in the combinations gamma-cyhalothrin-LPSc-1067, -LPSc-963, lambda-cyhalothrin-LPSc-1067, rynaxypyr-LPSc-1067, -LPSc-1098, and lufenuron-LPSc-1067 and antagonism with lambda-cyhalothrin-LPSc-1098, -LPSc-963, rynaxypyr-LPSc-963, lufenuron-LPSc-963, and methoxyfenozide-LPSc-907, -LPSc-963 added together; whereas in all other pairings of insecticide and fungus the effect was strictly additive (Table 4). At 25% maximum concentration, the lethality of the insecticides and fungal strains was usually additive in combination—except for the pairings gamma-cyhalothrin-LPSc-1067, lambda-cyhalothrin-LPSc-1067, rynaxypyr-LPSc-1067, and methoxyfenozide-LPSc-1082—where synergism occurred. At this lower concentration, the only combination in which we observed antagonism was with rynaxypyr-LPSc-1098 (Table 4). Finally, the pairing gamma-cyhalothrin-LPSc-1067 produced the highest mortality ($70 \pm 0.7\%$). Moreover, at both 50% and 25% of the maximum concentrations of both the insecticides and the fungal isolates, the combinations gamma-cyhalothrin-LPSc-1067 and lambda-cyhalothrin-LPSc-1067 produced the highest percent mortality on the larvae of *R. nu* (Fig. 1B, C).

Table 4 lists the toxicologic parameter LC_{50} determined after a 10-day exposure to each pesticide alone or in combination with one of the fungal strains. Gamma-cyhalothrin alone was the insecticide with the lowest lethal concentration at an LC_{50} of 20.6 ppm, whereas lufenuron manifested the highest at an LC_{50} of 100 ppm. In most instances, a lower LC_{50} was obtained when combining any of the five pesticides with one of the different fungal isolates, except for the following combinations: gamma-cyhalothrin-LPSc-1098 and gamma-cyhalothrin-LPSc-963, with LC_{50} values of 26.6 and 24.2 ppm, respectively; lambda-cyhalothrin-LPSc-907, lambda-cyhalothrin-LPSc-1098, and lambda-cyhalothrin-LPSc-963, with LC_{50} values of 61.9, 63.5, and 45.7 ppm, respectively, as opposed to 45.0 for the pesticide alone; and methoxyfenozide-LPSc-963 at an LC_{50} of 104 ppm, with the value for methoxyfenozide being 87.1 (Table 4). Furthermore, we found similar values for the LC_{10} and the NEC sublethal toxicologic parameters: those values were 11.4 and 9.7 ppm, respectively, for gamma-cyhalothrin; 40.6 and 39.7 ppm for lambda-cyhalothrin; 60 and 60 ppm for rynaxypyr; 100 and 100 ppm for lufenuron; and 69.8 and 64.31 ppm for methoxyfenozide.

The MST calculated for the strains, the insecticides, and their combinations at 100% concentrations indicated that the most virulent combinations were gamma-cyhalothrin-LPSc-1067 and lambda-cyhalothrin-LPSc-1067 with respective MSTs of 1.0 and 1.2 days, which survival

times were significantly different from those recorded after exposure to the rest of the pesticide treatments (Wilcoxon Test, p value <0.05). In contrast, the highest MSTs were observed with the strain LPSc 963 (8.1 days) and with the combinations methoxyfenozide-LPSc-1098 and lufenuron-LPSc-1098 (7.9 days), with those being significantly different from the MSTs recorded with the rest of the treatments (Wilcoxon Test, p value <0.05; Table 5). At a 50% maximum field concentration, the lowest MST was 4.6 days after exposure to gamma-cyhalothrin-LPSc-1067 and gamma-cyhalothrin-LPSc-1082 and 4.8 days in the presence of the combination lambda-cyhalothrin-LPSc-1067, which values were significantly different from those the other pesticide treatments (Wilcoxon Test, p value <0.05; Table 5). At a 25% concentration, the MST could be calculated for only the combinations gamma-cyhalothrin-LPSc-1067 and lambda-cyhalothrin-LPSc-1082, with those survivals accordingly being 7.3 and 8.3 days, respectively (Table 5).

Discussion

A combination of sublethal concentrations of chemical insecticides and entomopathogenic fungi can cause increased stress, immunocompromise, and a consequent alteration in insect physiology and behavior—there especially in survival and reproduction; thus leading to more favorable results in an insect-control program that includes such a biologic component⁶. A pairing of sublethal concentrations of insecticides with fungal entomopathogens can increase pest mortality as well as reduce the killing time compared to the use of either agent alone¹⁰. We observed that although some differences in conidial viability did occur in the presence of the five chemical pesticides investigated—with respect to the vegetative growth and conidia production of all fungal isolates—such differences were either low or insignificant. In many combinations, especially at low insecticide doses, the values were comparable to those of the controls. We observed differences in viability parameters due to toxic effect of the five chemical pesticides investigated in doses-dependent relationship. The vegetative growth of all strains of *B. bassiana* was not affected by lambda-cyhalothrin and gamma-cyhalothrin; and to a lesser extent by *M. anisopliae* strain LPSc 907. Also, the conidial production and viability of the five fungal isolates was less affected by gamma-cyhalothrin at concentrations lesser or equals to 50% of recommended dose. In all cases, lufenuron and methoxyfenozide had a greater deleterious effect on viability parameters analyzed. In a similar study Oliveira et al.²⁶ reported that formulations of

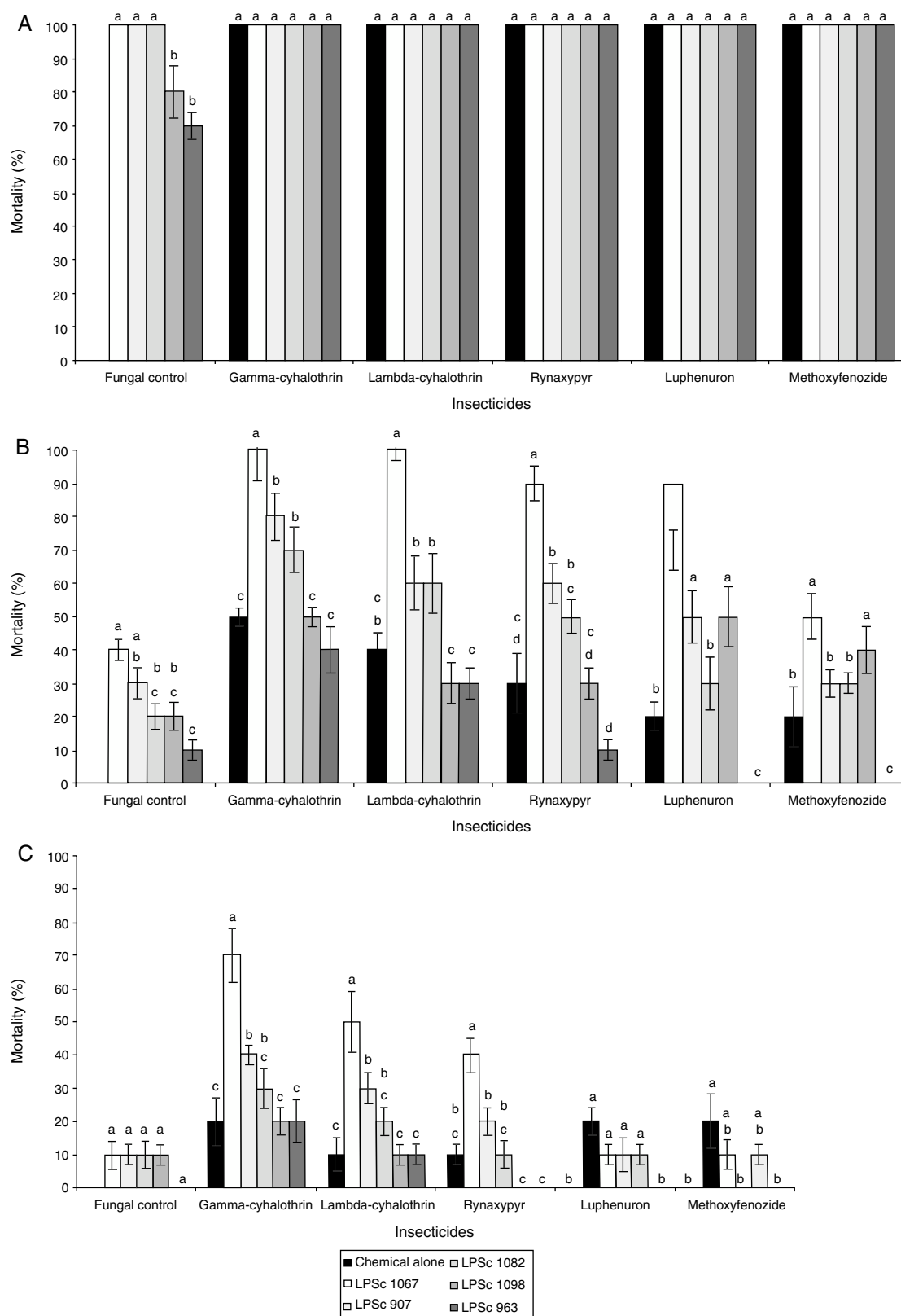


Figure 1 Mean percent $\pm$ SD mortality of *Rachiplusia nu* exposed to chemical insecticides and entomopathogenic fungi both alone and in combination at (a) the maximum field concentration (100%) and at concentrations of the chemical agents and fungal strains both reduced to (b) 50% and (c) 25%. The bars indicate standard errors, and different letters denote significant differences between fungal strains within each insecticide treatment according to the LSD test ($p \leq 0.05$).

Table 4 Median lethal concentrations (LC₅₀s) in ppm to *Rachiplusia nu* of chemical insecticides alone and in combination with the entomopathogenic fungal strains, after a 10-day exposure

Treatments	Toxicological parameters		Interaction χ^2 (response)*		
	LC ₅₀	SD	Field dose	50%	25%
Gamma-cyhalothrin	20.6	2.24	0.00 (additive)	–	–
Gamma-cyhalothrin + LPSc 1067	9.6	1.47	0.00 (additive)	11.3 (synergistic)	26.6 (synergistic)
Gamma-cyhalothrin + LPSc 907	14.7	0.79	0.00 (additive)	2.9 (additive)	2.14 (additive)
Gamma-cyhalothrin + LPSc 1082	15.7	0.78	0.00 (additive)	1.2 (additive)	0.24 (additive)
Gamma-cyhalothrin + LPSc 1098	26.6	1.78	0.00 (additive)	1.2 (additive)	0.94 (additive)
Gamma-cyhalothrin + LPSc 963	24.2	2.3	0.00 (additive)	27.6 (synergistic)	0.00 (additive)
Lambda-cyhalothrin	45	1.24	0.00 (additive)	–	–
Lambda-cyhalothrin + LPSc 1067	23.3	2.52	0.00 (additive)	16.8 (synergistic)	18.67 (synergistic)
Lambda-cyhalothrin + LPSc 907	61.9	46.4	0.00 (additive)	0.0 (additive)	0.73 (additive)
Lambda-cyhalothrin + LPSc 1082	32.9	2.3	0.00 (additive)	0.7 (additive)	0.01 (additive)
Lambda-cyhalothrin + LPSc 1098	63.5	34.1	0.00 (additive)	7.7 (antagonistic)	1.57 (additive)
Lambda-cyhalothrin + LPSc 963	45.7	3.95	0.00 (additive)	4.7 (antagonistic)	0.00 (additive)
Rynaxypyr	60	3.22	0.00 (additive)	–	–
Rynaxypyr + LPSc 1067	27.7	0.57	0.00 (additive)	12.6 (synergistic)	8.59 (synergistic)
Rynaxypyr + LPSc 907	23.3	1.65	0.00 (additive)	0.6 (additive)	0.01 (additive)
Rynaxypyr + LPSc 1082	28.1	2.08	0.00 (additive)	0.4 (additive)	1.57 (additive)
Rynaxypyr + LPSc 1098	35	2.63	0.00 (additive)	3.9 (synergistic)	7.03 (antagonistic)
Rynaxypyr + LPSc 963	40	2.06	0.00 (additive)	9.3 (antagonistic)	3.33 (additive)
Luphenuron	100	1.53	0.00 (additive)	–	–
Luphenuron + LPSc 1067	34.1	2.15	0.00 (additive)	3.8 (synergistic)	3.33 (additive)
Luphenuron + LPSc 907	46.6	3.62	0.00 (additive)	0.4 (additive)	0.00 (additive)
Luphenuron + LPSc 1082	56.1	5.41	0.00 (additive)	1.4 (additive)	0.00 (additive)
Luphenuron + LPSc 1098	52.4	4.55	0.00 (additive)	2.5 (additive)	0.00 (additive)
Luphenuron + LPSc 963	70.4	2.97	0.00 (additive)	11.6 (antagonistic)	0.00 (additive)
Methoxyfenozide	87.1	3.63	0.00 (additive)	–	–
Methoxyfenozide + LPSc 1067	72.1	2.91	0.00 (additive)	0.0 (additive)	3.33 (additive)
Methoxyfenozide + LPSc 907	80.9	8.65	0.00 (additive)	3.9 (antagonistic)	0.00 (additive)
Methoxyfenozide + LPSc 1082	82	7.06	0.00 (additive)	1.4 (additive)	152.1 (synergistic)
Methoxyfenozide + LPSc 1098	78.5	6.07	0.00 (additive)	0.2 (additive)	0.00 (additive)
Methoxyfenozide + LPSc 963	104	4.31	0.00 (additive)	11.6 (antagonistic)	0.00 (additive)

* The number of dead lepidopterans observed and the number of dead lepidopterans expected was used to test the hypothesis of independence with 1 df and $p=0.05$. Additivity was indicated if $\chi^2 < 3.84$, antagonism if $\chi^2 > 3.84$ and $P_c < P_e$, where P_c is the mortality observed with the insecticide plus the fungal strain and P_e is the expected mortality of the combination of the two agents. Synergism was indicated if $\chi^2 > 3.84$ and $P_c > P_e$. SD, standard deviation.

conventional insecticides exhibit a toxic effect on viability parameters of *B. bassiana* evaluated in this work. These authors report a great effect on vegetative growth and a variable effect on conidial production being in some cases compatibles with the tested insecticides; however, full recommended doses inhibit the conidial viability.

Although recent reports have indicated that various oil-based surfactants may influence the viability and virulence of fungal entomopathogens in several ways²¹, nevertheless studies aimed at investigating the *in vitro* compatibility between insecticides and entomopathogenic fungi have always had the advantage of exposing the fungal pathogen to the maximum action of the chemical compounds, a condition that often does not occur under field conditions. To the contrary, any compatibility indicated *in vitro* with an insecticide would be strong preliminary evidence in favor of the utility of that combination under field conditions². Moreover, those latter authors ascertained that conidial germination in the presence of commercial pesticides *in vitro* was one

of the most informative indicators of compatibility for use in the field. In the study reported here, with most of the insecticides at low doses, we observed that the *B. bassiana* strain LPSc 1067 was the least affected by the action of the five chemical insecticides since that strain obtained a higher percentage of viable conidia with no accompanying change in the vegetative growth and conidia production. In contrast, *M. robertsii* strain LPSc 963 had the lowest values for the percentage of viable conidia, vegetative growth, and conidia production. Similar results had been observed by Oliveira et al.²⁶, who reported that formulations of insecticides based on triazophos, chlorpyrifos, and endosulfan inhibited the viability of *B. bassiana* conidia by 100% at the three concentrations used. In this investigation, the five chemical insecticides exhibited varying interactions (*i.e.*, synergism, additivity, or antagonism) with respect to the toxicity against *R. nu* larvae when combined with the strains *B. bassiana* LPSc 1067, 1082, 1098; *M. anisopliae* LPSc 907; or *M. robertsii* LPSc 963; with mostly additivity being observed.

Table 5 Median survival time in days ($\pm$ SD) at different concentrations of fungi and pesticides alone or in combination

Treatments	Dose [*]		
	100%	50%	25%
LPSc 1067	5.1 $\pm$ 0.50 f g h	–	–
LPSc 1067 + gamma-cyhalothrin	1 $\pm$ 0.0 a	4.6 $\pm$ 0.29 b	7.3 $\pm$ 0.41 a
LPSc 1067 + lambda-cyhalothrin	1.2 $\pm$ 0.07 a	4.8 $\pm$ 0.58 b	8.3 $\pm$ 0.43 a
LPSc 1067 + rynaxypyr	2.1 $\pm$ 0.1 b c	5.8 $\pm$ 0.30 c d	–
LPSc 1067 + luphenuron	2.5 $\pm$ 0.22 c d	6.6 $\pm$ 0.51 d e	–
LPSc 1067 + methoxyfenocide	3.6 $\pm$ 0.26 e	8.5 $\pm$ 0.38 g h	–
LPSc 907	6.1 $\pm$ 0.54 i j	–	–
LPSc 907 + gamma-cyhalothrin	1.5 $\pm$ 0.09 a b	5.3 $\pm$ 0.60 b c	–
LPSc 907 + lambda-cyhalothrin	1.8 $\pm$ 0.16 a b c	5.5 $\pm$ 0.56 b c	–
LPSc 907 + rynaxypyr	3.7 $\pm$ 0.37 e	7.9 $\pm$ 0.57 f g	–
LPSc 907 + luphenuron	6.4 $\pm$ 0.46 i j	7.8 $\pm$ 0.37 f g	–
LPSc 907 + methoxyfenocide	7.3 $\pm$ 0.48 j k	–	–
LPSc 1082	6.7 $\pm$ 0.59 j	–	–
LPSc 1082 + gamma-cyhalothrin	2.2 $\pm$ 0.21 b c	4.6 $\pm$ 0.47 b	–
LPSc 1082 + lambda-cyhalothrin	3.4 $\pm$ 0.32 d e	7.2 $\pm$ 0.62 e f	–
LPSc 1082 + rynaxypyr	5.7 $\pm$ 0.57 g h i	8.2 $\pm$ 0.49 g	–
LPSc 1082 + luphenuron	6.2 $\pm$ 0.64 i j	9.4 $\pm$ 0.26 h	–
LPSc 1082 + methoxyfenocide	5.7 $\pm$ 0.56 g h i	9.4 $\pm$ 0.29 h	–
LPSc 1098	7.7 $\pm$ 0.52 k	–	–
LPSc 1098 + gamma-cyhalothrin	4.7 $\pm$ 0.35 f	5.8 $\pm$ 0.35 c d	–
LPSc 1098 + lambda-cyhalothrin	5.6 $\pm$ 0.63 f g h i	6.8 $\pm$ 0.34 e	–
LPSc 1098 + rynaxypyr	5.9 $\pm$ 0.50 h i	–	–
LPSc 1098 + luphenuron	7.9 $\pm$ 0.34 k	–	–
LPSc 1098 + methoxyfenocide	7.9 $\pm$ 0.42 k	–	–
LPSc 963	8.1 $\pm$ 0.48 k	–	–
LPSc 963 + gamma-cyhalothrin	5.1 $\pm$ 0.59 f g h	–	–
LPSc 963 + lambda-cyhalothrin	5.6 $\pm$ 0.53 f g h i	–	–
LPSc 963 + rynaxypyr	6.1 $\pm$ 0.55 i j	–	–
LPSc 963 + luphenuron	7.2 $\pm$ 0.37 j k	–	–
LPSc 963 + methoxyfenocide	7.8 $\pm$ 0.35 k	–	–
gamma-cyhalothrin	1.2 $\pm$ 0.07 a	1.9 $\pm$ 0.04 a	–
lambda-cyhalothrin	1.3 $\pm$ 0.11 a	–	–
rynaxypyr	1.8 $\pm$ 0.16 a b c	–	–
luphenuron	2.3 $\pm$ 0.22 b c	–	–
methoxyfenocide	4.9 $\pm$ 0.38 f g	–	–

* Different letters denote significant differences between treatments in the same column according to the Wilcoxon test ($p < 0.05$).

In a total of 10 pairings, however, synergistic responses did occur at either 50% or 25% of the maximum field concentrations of both the chemical insecticides and the fungal isolates, while in 7 combinations we recorded antagonistic effects (Table 4). Based on these observations, certain combinations of the chemical pesticides and entomopathogenic fungi that we tested in this work were particularly effective against *R. nu* larvae at reduced doses (50% and 25%) because of the occurrence of such synergistic interactions between those agents.

Various authors have reported differing results in the combined action of fungal strains and chemical insecticides in field applications. For example, the combination *B. bassiana*–DimilinTM was more effective than a pairing of *B. bassiana* with other chemical agents in field applications against grasshoppers in the United States¹⁰. In populations of grasshoppers in Mali treated with a mixture of *B. bassiana* and DimilinTM, Delgado et al.⁸ observed that

the decrease in the numbers of insects continued until the end of the monitoring. The so-called *insect-growth regulators* (IGRs)—i.e., chemical insecticides known to be less detrimental to beneficial insects and more worker-friendly because of an action not involving the insect nervous system—are more environmentally safe than conventional insecticides. For this reason, in recent years, IGRs have been favored for use in integrated-pest-management (IPM) programs³². In the present work, the IGR insecticides luphenuron and methoxyfenozide produced a high mortality at maximum field doses, although at doses reduced to 50% and 25% of those maximum doses lower mortalities were recorded than those documented for the other insecticides. In contrast, the combination gamma-cyhalothrin–LPSc-1067 not only caused the highest percent mortality against *R. nu* larvae among all the combinations tested but also exhibited a synergistic interaction at 50% and 25% of the maximum field doses. Furthermore, the

combinations gamma-cyhalothrin-LPSc-1067 and lambda-cyhalothrin-LPSc-1067 produced a similar percent mortality of *R. nu* larvae, even at doses of 50% of the maximum.

The pairing of the insecticides gamma-cyhalothrin and lambda-cyhalothrin, in the formulations and doses tested, had no deleterious effects on the percentage of viable conidia, vegetative growth, or conidia production of *B. bassiana* strain LPSc 1067. In addition, this fungal isolate combined with those pesticides produced the highest percent mortality of *R. nu* larvae. Therefore, these pesticide-fungus pairings can be recommended for an IPM program aimed at the control of *R. nu*. However, one of the major challenges for the success of a fungal entomopathogen in the field is the development of an adequate formulation. The fungal formulation process must consider several objectives. Foremost is the stabilization of the organism to allow prolonged storage. In addition, the formulation must be suitable for handling and release in the field, and protect the entomopathogenic fungi from adverse environmental factors.

Future laboratory studies will be needed using *B. bassiana* LPSc 1067, to the aim of obtaining a stable fungal formulation, before it can be evaluated in combination with the chemical insecticides gamma-cyhalothrin and lambda-cyhalothrin on field trials, for an IPM control of the lepidopteran pests of soybean, *R. nu*.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This study was partially supported by Consejo Nacional de Investigaciones Científicas y Tecnológicas (PIP 0018), Universidad Nacional de La Plata (UNLP, 11/N 651) and Fondo para la Investigación Científica y Tecnológica (FONCYT; PICT 2013-0543; PICT2015-1146). Dr. Donald F. Haggerty, a retired academic career investigator and native English speaker, edited the final version of the manuscript.

References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol.* 1990;215:403–10.
- Alves SB, Moino A, Almeida JEM. Produtos fitossanitários e entomopatógenos. In: Alves SB, editor. Controle microbiano de insetos, Piracicaba FEALQ. 1998. p. 217–38.
- Bidochka MJ, Kamp AM, Lavender TM, Dekoning J, De Croos JNA. Habitat association of two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: uncovering cryptic species? *Appl Environ Microbiol.* 2001;67:1335–42.
- Bischoff JF, Rehner SA, Humber RA. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia.* 2009;101:512–30.
- Bitsadze N, Jaronski S, Khasdan V, Abashidze E, Abashidze M, Latchinsky A, Samadashvili D, Sokhadze I, Rippa M, Ishaaya I, Horowitz AR. Joint action of *Beauveria bassiana* and the insect growth regulators diflubenzuron and novaluron, on the migratory locust, *Locusta migratoria*. *J Pest Sci.* 2013;86:293–300.
- Boucias DG, Stokes C, Storey G, Pendland JC. The effect of imidacloprid on the termite *Reticulitermes flavipes* and its interaction with the mycopathogen *Beauveria bassiana*. *Pflanzenschutz-Nachr Bayer.* 1996;49:103–44.
- CASAFE. Argentina chamber of agricultural health and fertilizers guide pesticides, vol. 1. Buenos Aires, Argentina.; 2013.
- Delgado FX, Britton JH, Onsager JA, Swearingen W. Field assessment of *Beauveria bassiana* (Balsamo) Vuillemin and potential synergism with Diflubenzuron for control of Savanna grasshoppers complex (Orthoptera) in Mali. *J Invertebr Pathol.* 1999;73:34–9.
- FAO. The control pests in agriculture Argentina. Rural agriculture sector study. Argentina: World Bank/Investment Centre FAO; 2005.
- Foster RN, Reuter KC, Black L, Britton J. Evaluation of the fungus *Beauveria bassiana* with selected insecticide stressors for control of unconfined rangeland grasshoppers. *Arthropod Management Tests.* 1996;21:280.
- Genstat 12th edition. Statistical software. UK: VSN International; 2009.
- Goettel M, Inglis GD. Fungi: Hyphomycetes. In: Lacey L, editor. Manual of techniques in insect pathology. San Diego, California: Academic Press; 1997. p. 213–48.
- Gonzalez M, Miglioranza KS, Aizpún JE, Isla FL, Peña A. Assessing pesticide leaching and desorption in soils with different agricultural activities from Argentina (Pampa and Patagonia). *Chemosphere.* 2010;81:351–8.
- Greene GL, Leppla NC, Dickerson WA. Velvetbean caterpillar: a rearing procedure and artificial medium. *J Econ Entomol.* 1976;69:487–8.
- Hunter DM, Milner RJ, Spurgin PA. Aerial treatment of the Australian plague locust, *Chortiocetes terminifera* (Orthoptera: Acrididae), with *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) in Australia. *Bull Entomol Res.* 2001;91:93–9.
- INTA; 2015. Instituto Nacional de Tecnología Agropecuaria. <http://inta.gob.ar/sites/default/files/inta-usda-noviembre-2015.pdf>
- Jager T, Heugens EHW, Kooijman SALM. Making sense of ecotoxicological test results: towards application of process-based models. *Ecotoxicology.* 2006;15:305–14.
- Jaronski ST. Ecological factors in the inundative use of fungal entomopathogens. *BioControl.* 2010;55:159–85.
- Lacey LA, Brooks WM. Initial handling and diagnosis of diseased insects. In: Lacey LA, editor. Manual of techniques in insect pathology. San Diego, California: Academic Press; 1997. p. 1–15.
- Lomer CJ, Bateman RP, Johnson DL, Langewald J, Thomas M. Biological control of locust and grasshoppers. *Annu Rev Entomol.* 2001;44:667–702.
- Mascarin GM, Kobori NN, Quintela ED, Arthurs SP, Júnior ID. Toxicity of non-ionic surfactants and interactions with fungal entomopathogens toward *Bemisia tabaci* biotype B. *BioControl.* 2014;59:111–23.
- MAGyP Ministry of Agriculture. Livestock and fisheries of the nation; 2014 http://www.siaa.gov.ar/_apps/siaa/arboreal/total_pciaX.php?respuesta=AG
- Neves PMOJ, Hirose E, Tchujo PT, Moino A. Compatibility of entomopathogenic fungi with neocotinoid insecticides. *Neotrop Entomol.* 2001;30:263–8.

24. Nishimatsu T, Jackson JJ. Interaction of insecticides, entomopathogenic nematodes, and larvae of the western corn rootworm (Coleoptera: Chrysomelidae). *J Econ Entomol.* 1998;91:410–8.
25. Nussenbaum AL, Lecuona RE. Selection of *Beauveria bassiana* sensu lato and *Metarhizium anisopliae* sensu lato isolates as microbial control agents against the boll weevil (*Anthonomus grandis*) in Argentina. *J Invertebr Pathol.* 2012;110:1–7.
26. Oliveira CN, Neves MO, Kawazoe LS. Compatibility between the entomopathogenic fungus *Beauveria bassiana* and insecticides used in coffee plantations. *Sci Agricola.* 2003;60:663–7.
27. Pelizza SA, Eliades LA, Scorsetti AC, Cabello MN, Lange CE. Entomopathogenic fungi from Argentina for the control of *Schistocerca cancellata* (Orthoptera: Acrididae) nymphs: fungal pathogenicity and enzyme activity. *Biocontrol Sci Technol.* 2012;22:1119–29.
28. Pelizza SA, Eliades LA, Saparrat MCN, Cabello MN, Scorsetti AC, Lange CE. Screening of Argentine native fungal strain for biocontrol of the grasshoppers *Tropidacris collaris*: relationship between fungal pathogenicity and chitinolytic enzyme activity. *World J Microbiol Biotechnol.* 2012;28:1359–66.
29. Péry AR, Flammarion P, Vollat B, Bedaux JJ, Kooijman SA, Garric J. Using a biology-based model (DEBtox) to analyze bioassays in ecotoxicology: opportunities and recommendations. *Environ Toxicol Chem.* 2002;21:459–65.
30. Rehner SA, Buckley E. A *Beauveria* phylogeny inferred from nuclear ITS and EF1- α sequences: evidence for cryptic diversification and links to *Cordyceps teleomorphs*. *Mycologia.* 2005;97:84–98.
31. Rimoldi F, Fogel MN, Schneider MI, Ronco AE. Lethal and sub-lethal effects of cypermethrin and methoxyfenozide on the larvae of *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae). *Invertebr Repr Dev.* 2012;56:202–8.
32. Schneider M, Smagghe G, Viñuela E. Susceptibility of *Hyposoter didymator* (Hymenoptera: Ichneumonidae) adults to several IGRs pesticides and spinosad by different exposure methods. *IOBC/WPRS Bull.* 2003;26:111–22.
33. Stenglein S, Balatti P. Genetic diversity of *Phaeoisariopsis griseola* in Argentina as revealed by pathogenic and molecular markers. *Physiol Mol Plant Pathol.* 2006;68:158–67.
34. Vega FE, Meyling NV, Luangsa-Ard JJ, Blackwell M. Fungal entomopathogens. In: Vega FE, Kaya HK, editors. *Insect pathology*. London: Elsevier; 2012. p. 171–220.
35. Xlstat life software; 2013. Addinsoft SARL Paris, France. <http://www.xlstat.com>