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RESEARCH ARTICLE

Use of entomopathogenic fungi combined with biorational insecticides to control *Dichroplus maculipennis* (Orthoptera: Acrididae: Melanoplinae) under semi-field conditions

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Grasshoppers are among the invertebrate herbivores that cause most economic losses in grasslands throughout Argentina's Pampas and parts of Patagonia. Chemical insecticides remain the sole option for grasshopper control in this area, despite being of significant environmental concern. Our aim was to evaluate the efficacy of combinations between three entomopathogenic fungi strains (*Beauveria bassiana* LPSc 1067 and LPSc1082), and *Metarhizium anisopliae* (LPSc 907), two biorational insecticides (luphenuron and methoxyfenozide), and a new synthetic chemical pesticide (rynaxypyr) in the control of the pest grasshopper *Dichroplus maculipennis* under field cage conditions. Fungal strains used were adjusted to 1×10^8, 1×10^6 and 1×10^4 conidia/ml. Insecticides were tested at three concentrations: the average concentration recommended for application in the field (100%), 50% of that level and finally 25%. Combinations of the insecticides with *B. bassiana* (LPSc 1067, LPSc 1082) and *M. anisopliae* (LPSc 907) caused higher mortality to *D. maculipennis* nymphs than any of the individual agents used alone. The three insecticides tested did not negatively affect the isolates of the two species of entomopathogenic fungi employed.

Keywords: entomopathogenic fungi; grasshoppers; biorational insecticides; Beauveria bassiana; Metarhizium anisopliae

1. Introduction

Dichroplus maculipennis (Blanchard) is one of the main grasshopper pests in Argentina, causing damage in grasslands and in economically important crops such as maize, soybean and wheat. This pest occurs in Argentina (Buenos Aires, Chubut and Neuquén provinces), Uruguay, Chile and Brazil in South America (Carbonell, Cigliano, & Lange, 2006; Cigliano, Pocco, & Lange, 2014; Mariottini, De Wysiecki, & Lange, 2011a). In recent years an outbreak (50 ind/m^2) occurred in southern Buenos Aires province, causing an important economic damage to farmers and ranchers (Mariottini, De Wysieki, & Lange, 2011b).

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To date, chemical insecticides are the only available option for grasshopper control in Argentina, but their use is of serious environmental concern (Gonzalez, Miglioranza, Aizpún, Isla, & Peña, 2010). On the other hand, chemical insecticides, given their mode of action, cause damage also on non-target insect fauna. Thus, alternatives to these insecticides must be found. One of these promising alternatives are insect growth regulators (IGRs) as they disrupt the insect's normal metabolic processes, causing alterations in metamorphosis, development and reproduction (El-Sayed, El-Sheikh, & Mohamed, 2011; Kai, Huang, Tobe, & Yang, 2009). Chitin synthesis inhibitors (CSIs) such as hexaflumuron, luphenuron and diflubenzuron inhibit the production of chitin, therefore disrupting the normal moulting process.

Other alternatives that have attracted increasing interest for the control of pest insects are the entomopathogenic fungi *Metarhizium anisopliae* (Metschn.) Sorokin and *Beauveria bassiana* (Bals.-Criv) Vuillemin (Ascomycota: Hypocreales) because of their specificity and environmental safety (Nussenbaum & Lecuona, 2012). However, a criticism raised in relation to the use of entomopathogenic fungi as biocontrol agents is that the microbial action is too slow (Lomer, Bateman, Johnson, Langewald, & Thomas, 2001). Another drawback mentioned in the use of entomopathogenic fungi alone to control grasshoppers and locusts is that it has been found that during a fungal infection the Desert locust *Schistocerca gregaria* (Forskal) behaviourally fevers by seeking out higher environmental temperatures than their healthy conspecifics (Bundey et al., 2003; Elliot, Blanford, & Thomas, 2002). The alternative use of chemical insecticides – ideally at low, sublethal levels – in combination with entomopathogenic fungi was developed as an attractive approach to circumvent these difficulties (Bitsadze et al., 2013).

Therefore, the sustainable agriculture is nowadays based on integrated pest management which contemplates the use of different chemical insecticides and entomopathogenic fungi in a joint manner (Purwar & Sachan, 2006). In this article, we evaluated the interactions between three strains of entomopathogenic fungi and three insecticides (two of them considered biorational) under semi-field cage conditions in order to determine the possible usefulness of combinations of these agents against the pest grasshopper *D. maculipennis*.

2. Materials and methods

2.1. Insect

Individuals of *D. maculipennis* used in this study belonged to the first laboratory generation [F1] of specimens originally collected in the southern Pampas regions (Laprida county, Buenos Aires province, 37°32′60″S, 60°49′00″), and maintained in a rearing room under controlled conditions (30°C, 14L: 10D, 40% RH) as described in previous studies (De Wysiecki, Cigliano, & Lange, 1997; Mariottini et al., 2011b).

2.2. Insecticides and fungal strains

The synthetic chemical pesticide Coragen™ (rynaxypyr 20% [w/v], DuPont S.A., Argentina) and two biorational chemical insecticides Match™ (luphenuron 5% [w/v], Sygenta S.A., Argentina) and Intrepid™ (methoxyfenozide 24% [w/v], Dow AgroSciences S.A., Argentina) were used. Rynaxypyr acts as an activator of the ryanodine receptors of insects, undermining the process of muscle contraction.

Affected individuals suffer paralysis and quickly stop feeding. Luphenuron acts by inhibiting the growth of insects and interfering with chitin synthesis. Methoxyfenozide acts on the growth hormone of insects accelerating the moulting process. The insecticides were tested at three concentrations: the average concentration recommended for application in the field on soybean (100%), 50% and finally 25% of this level, giving concentrations 60, 30 and 15 ppm (mg a.i./l) for rynaxypyr; 100, 50 and 25 ppm (mg a.i./l) for luphenuron; and 144, 72 and 36 ppm (mg a.i./l) for methoxyfenozide. The fungal strains used were the Spegazzini-Institute–culture-collection LPSc 1067 and LPSc 1082 of *B. bassiana* and strain LPSc 907 of *M. anisopliae*. All isolates were preserved by lyophilisation. Morphological species identification was corroborated by extracting the DNA of the monosporic cultures according to Stenglein and Balatti (2006). PCRs were carried out in an XP thermal cycler (Bioer Technology Co, Hangzhou, China) to amplify and sequence the ITS rDNA region of *B. bassiana* using primer pairs ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′)/ITS4 (5′-TCC TCC GCT TAT TGA TATGC-3′) (White, Bruns, Lee, & Taylor, 1990) and the translation elongation factor 1-alpha (EF intron region) of *M. anisopliae* using primer pairs EF1T (5′-ATG GGT AAG GAR GAC AAG AC-3′)/EF2T (5′-GGA AGT ACC AGT GAT CAT GTT-3′) (Bischoff, Rehner, & Humber, 2006, 2009; Rehner & Buckley, 2005). Fragments similarities with previously published sequence data were examined with BLASTn (Altschul, Gish, Miller, Myers, & Lipman, 1990) in the NCBI web page. The sequences generated in this study were submitted to GenBank (accession numbers KF500409 and KJ7722495 for *B. bassiana* LPSc 1067 and LPSc 1082, respectively; KJ772494 for *M. anisopliae* LPSc 907). The choice of these fungal strains was based on their laboratory efficacy against other pest grasshopper and locust species from Argentina (Pelizza, Elíades, Saparrat, et al., 2012; Pelizza, Elíades, Scorsetti, Cabello, & Lange, 2012). Conidia of the fungal strains were obtained from cultures on potato-dextrose-agar medium (Britania S.A., Buenos Aires, Argentina) after incubation for 10 days at 25°C in the dark. Afterward, conidia were harvested with disposable cell scrapers (Fisherbrand™) and placed in test tubes containing 0.01% (v/v) polyoxyethylene sorbitan monolaurate (Tween 80™; Merck). 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 Inglis (1997). This germination test was repeated for each stock suspension to maintain the constancy of the viability assessments. In all cases, the average viability of the conidia was over 95%.

2.3. Interaction between entomopathogenic fungi and chemical insecticides against **D. maculipennis**

Experimental inoculations *per os* were conducted on third-instar nymphs of *D. maculipennis* in order to determine the percent mortality at each concentration of the insecticides and the fungal strains alone and combined. We chose this inoculation technique on the basis of its recommendation by the manufacturer of all three chemical insecticides. Twenty-four hours before the beginning of the experiment, nymphs were placed individually in 20-ml foam-plugged glass vials and left without food. For the combined treatments, 5 ml of each fungal suspension and each

insecticide solution were combined in the proper concentration to obtain final solutions with the concentration specified above. Suspensions were homogenised by vortexing for 2 min. For the non-combined treatments (chemicals and fungal strains alone) 10 ml of a solution with the proper concentration were prepared. Lettuce disks (diameter, 1 cm) were submerged in the solutions and then given to treated groups according to Inglis, Johnson, and Goettel (1996). Control groups were fed with lettuce disks submerged in 0.01% (v/v) Tween 80™. Under each experimental condition, three replicates (on different dates) of 10 third-instar nymphs each were processed for both the treated and control insects. Immediately after ingestion of the whole lettuce disk, treated and control nymphs were placed in wire-screened, aluminium cages (70 × 50 × 50 cm) as groups of 10 individuals under field conditions. The cages were located in a field near the city of Brandsen (Buenos Aires province) (S 35°04′44.4″; WO 58°08′26.7″) at an altitude of 22 (m.a.s.l.). A mixture of grasses *Bromus* and *Lolium* predominantly covered the site. The cumulative mortality was recorded daily for 10 days. Dead grasshoppers with no external mycelium were surface-sterilised by dipping them successively in 70% ethanol (10–15 s), 0.5% sodium hypochlorite solution (1 min) and sterile distilled water (1 min, two consecutive baths) according to Lacey and Brooks (1997). Next, they were placed in a sterile culture chamber consisting of a Petri dish (60 mm diameter) with a filter-paper disk that was periodically moistened with sterile distilled water and incubated at 25°C in the dark. Mycosis was confirmed by microscopic examination of dead grasshoppers.

Maximum and minimum temperature and relative humidity was recorded daily in the site where the test was performed. The temperature average registered during the trials fluctuated between a maximum of 31.1°C and a minimum of 16.8°C, the relative humidity averaged was 56.3%.

2.4. Data analysis

When the mortality in the treated grasshoppers was 50% or higher, the median survival time (MST) was calculated based on Kaplan–Meier Survival distribution function (XLSTAT Life Software, 2013). Pairwise comparisons between survival curves were made by log-rank test. In addition, when the mortality in the grasshoppers treated was 50% or higher, the following lethal and sublethal-toxicity parameters were calculated: the median lethal concentration (LC50), the lethal concentration to 10% of the exposed individuals (LC10) using EPA probit analysis program, version 1.5 (Finney, 1971; Fisher & Yates, 1963) and the non-effect concentration (NEC) by means DEBtox v. 2.0.1 program (Jager, Heugens, & Kooijman, 2006; Péry et al., 2002). The LC50, LC10 and NEC values were expressed as parts per million and the MST in days.

Significant differences between the percent mortalities after a 10-day exposure to the different treatments were analysed using the Wald Statistics (generalised linear model) with the Genmod Prod-function with the software SAS (2001) with a binomial error distribution and logit link function. An a-posteriori test Fisher's LSD test with Statistica 7 software led us to detect significant differences between treatments.

Analysis for additive, antagonistic or synergistic interaction was based on a binomial test and comparison of the expected and observed percentage mortality

according to Nishimatsu and Jackson (1998). Expected mortality at a set concentration of insecticide and fungal strain was based on the formula $P_e = P_o + (1 - P_o)(P_1) + (1 - Po)(1 - P_1)(P_2)$, where P_e is the expected mortality on combination of the two insecticidal agents, P_o is the natural (control) mortality, P_1 is the mortality after treatment with the insecticide alone, and P_2 is the mortality after treatment with the fungal strain alone. The chi-square statistic was calculated as $\chi^2 = (L_o - L_e)^2/L_e + (D_o - D_e)^2/D_e$, where L_o is the number of living nymphs observed, L_e the number of living nymphs expected, D_o the number of dead nymphs observed, and D_e the number of dead nymphs expected. 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 after treatment with the insecticide and fungal strain combination and P_e is the expected mortality calculated with the formula described above. Synergism was indicated if $\chi^2 > 3.84$ and $P_c > P_e$.

3. Results

When we compared the percent mortality of third-instar *D. maculipennis* nymphs after a 10-day exposure, significant differences were found between variable doses, strains and for the interaction doses × strains in all cases except for the insecticide methoxyfenozide where interaction doses × strains was not significant ($\chi^2 = 6.08$; df = 4; *P*-value = 0.1931) (Table 1).

At 100% concentration, the non-combined treatments that produced the highest mortalities after 10-day exposure were strain LPSc 907 (83.33 ± 3.74%) followed by the insecticide rynaxypyr (73.33 ± 1.9%). The fungal strain LPSc 1082 and the chemical insecticide methoxyfenozide were the agents causing the lower percent mortality (56.6 ± 1.76 and 40 ± 2.62, respectively) (Figure 1). On the other hand, all combined treatments produced 100% mortality in the nymphs (Figure 1a).

At 50% of the maximum concentrations of both the chemical insecticides and the fungal isolates, we also observed an increase in the mortality of the *D. maculipennis* nymphs after exposure to the two agents in combination compared to the non-combined treatments. The combination rynaxypyr-LPSc907 produced the highest mortality (100%), whereas combination methoxyfenozide-LPSc1082 produced the lowest mortality (50 ± 3.3%). The percent mortalities caused by the fungal strains (*B. bassiana* LPSc 1067 and *M. anisopliae* LPSc 907 were higher than the values recorded for any of the chemicals alone, except for rynaxypyr that produced a higher

Table 1. Results of Wald Statistics (generalised linear model) on the percent mortalities of third-instar *Dichroplus maculipennis* nymphs after 10-day exposure at different concentrations of biorational pesticides alone and in combination with the *Beauveria bassiana* 1067, 1082 and *Metarhizium anisopliae* 907 strains.

	Rynaxypyr			Luphenuron			Methoxyfenozide		
Insecticides	df	χ^2	*P*-value	df	χ^2	*P*-value	df	χ^2	*P*-value
Doses	2	260.12	<0.0001	2	260.12	<0.0001	2	404.01	<0.0001
Strains	2	19.12	<0.0001	2	19.12	<0.0001	2	27.36	<0.0001
Doses × Strains	4	16.79	0.0021	4	16.79	0.0021	4	6.08	0.1931

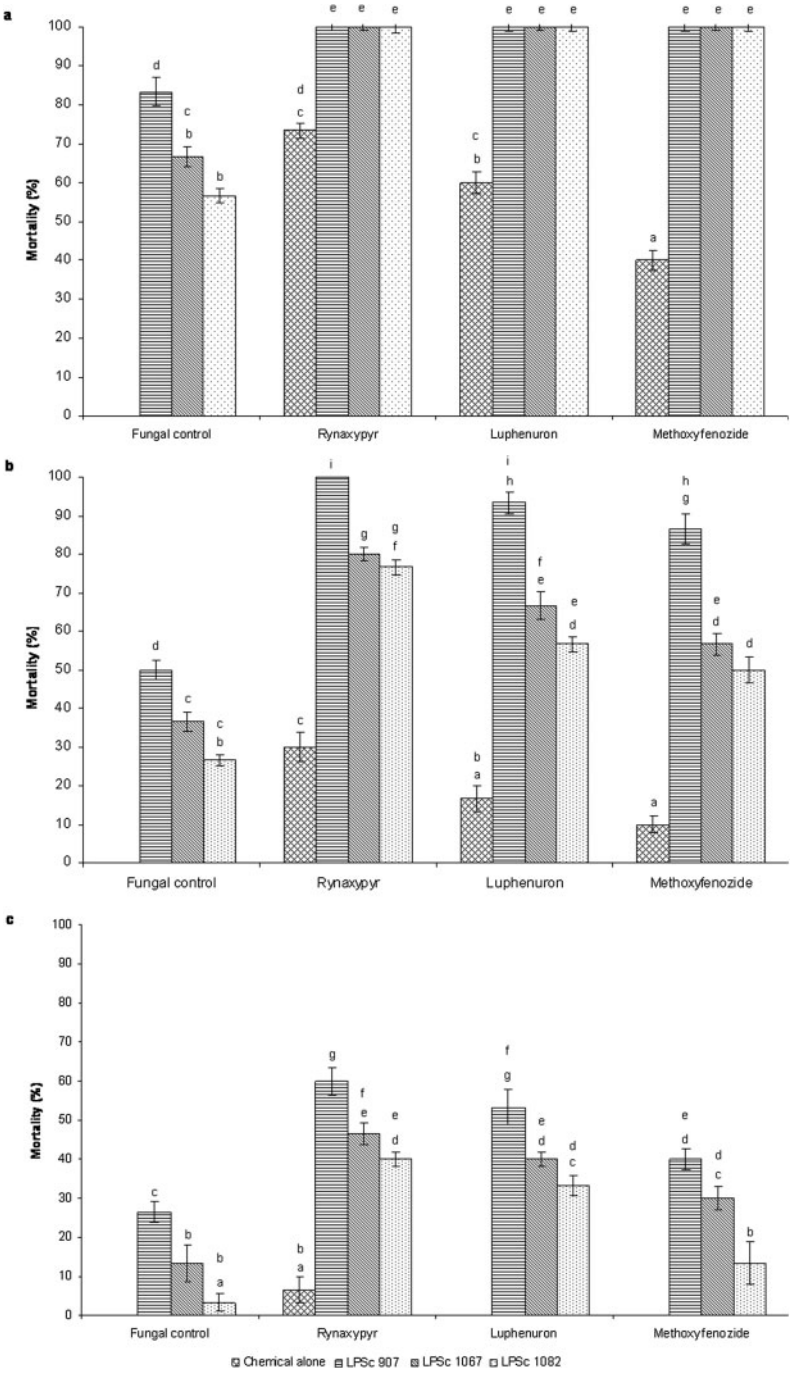

Figure 1. Mean percent ± SD mortality of *Dichroplus maculipennis* exposed to chemical insecticides and entomopathogenic fungi both alone and in combination at (a) maximum field concentration (100%) and at concentrations of the chemical agents and fungal strains reduced to (b) 50% and (c) 25%. Different letters denote significant differences between treatments according to the Fisher's LSD test ($P < 0.05$).

Table 2. Median survival time in days (±SD) at different concentrations of fungi and pesticides alone and in combination with the *Beauveria bassiana* 1067, 1082 and *Metarhizium anisopliae* 907 strains.

	Dose		
Treatments	100%	50%	25%
LPSc 907	7 ± 0.3 af	9.6 ± 0.11 a	–
LPSc 907 + rynaxypyr	2.9 ± 0.3 b	6.3 ± 0.33 b	8.3 ± 0.36 a
LPSc 907+ luphenuron	5 ± 0.4 c	7.4 ± 0.41 c	9 ± 0.24 a
LPSc 907 + methoxyfenozide	6.8 ± 0.37 adf	8.2 ± 0.33 cd	–
LPSc 1082	8.8 ± 0.31 e	–	–
LPSc 1082 + rynaxypyr	6.3 ± 0.38 fg	8.3 ± 0.30 cd	–
LPSc 1082 + luphenuron	6.4 ± 0.38 afg	9.4 ± 0.18 a	–
LPSc 1082 + methoxyfenozide	7.6 ± 0.23 a	9.9 ± 0.05 a	–
LPSc 1067	8.6 ± 0.25 e	–	–
LPSc 1067 + rynaxypyr	4.5 ± 0.43 c	7.2 ± 0.39 c	–
LPSc 1067 + luphenuron	5.7 ± 0.48 cdg	7.9 ± 0.43 ce	–
LPSc 1067 + methoxyfenozide	7 ± 0.41 afg	8.7 ± 0.31 ade	–
Rynaxypyr	6.9 ± 0.42 afg	–	–
Luphenuron	6.6 ± 0.29 afg	–	–
Methoxifenozide	8.5 ± 0.44 e	–	–

Different letters denote significant differences between treatments in the same column according to the Log-rank test ($P < 0.05$).

percent mortality that strain LPSc 1082 (30 ± 3.61% and 26.6 ± 1.6%, respectively) (Figure 1b).

At 25% of maximum concentrations we observed that the only chemical insecticide that caused mortality on *D. maculipennis* nymphs was rynaxypyr (6.6 ± 3.37%). Among the fungal strains alone, LPSc 907 produced the highest mortality (26.6 ± 2.63%) and LPSc 1082 the lowest (3.3 ± 2.2%). Again, the joint action of fungal strains and chemical insecticides produced the highest mortality. The combination of rynaxypyr–LPSc907 caused the highest mortality (60 ± 3.67%), whereas combination of methoxyfenozide-LPSc1082 produced the lowest mortality (13.3 ± 5.3%) (Figure 1c).

The MST calculated for strains LPSc 1067, LPSc 1082 and LPSc 907 showed that *M. anisopliae* LPSc 907 was the most virulent with values of 7 ± 0.3 and 9.6 ± 0.11 days for 100% and 50% of full dose, respectively. On the other hand, strains LPSc 1067 and LPSc 1082 led to values of 8.6 ± 0.25 and 8.8 ± 0.31 days for full dose (Table 2). We observed a decrease in the MST when complementing chemical insecticides with fungal strains. In all cases we observe that the lower values of MST were obtained when combining strain LPSc 907 with different chemical insecticides, followed by strain LPSc 1067 and finally strain LPSc 1082, which produced the highest MST values (Table 2). The lower value of MST was obtained with the combination rynaxypyr–LPSc907 (at the higher dose of 60 ppm) with 2.9 ± 0.3 days and the higher MST was observed in the combination methoxyfenozide–LPSc1082 (at 72 ppm) with 9.9 ± 0.05 days (Table 2).

Table 3. Toxicological parameters calculated by probit analysis: median lethal concentration (CL50), 95% confidence limits, slope and χ^2 for rynaxypyr, luphenuron and methoxyfenozide alone and in combination with the entomopathogenic fungal strains 907, 1067 and 1082 against *D. maculipennis* after a 10-day exposure. Interaction was based on χ^2 ratio of expected:observed mortality. 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 and P_e is the expected mortality of the combination. Synergism was indicated if $\chi^2 > 3.84$ and $P_c > P_e$. NC, non-calculated; SD, standard deviation

	Toxicological parameters					Interaction χ^2(response)		
Treatments	LC_{50}	LC 95%	Slope	SD	χ^2 calculated	Field dose	50%	25%
LPSc 1067	7.64×10^6	1.41×10^6–9.06×10^7	0.39	0.09	0.00	–	–	–
LPSc 1082	3.52×10^7	7.69×10^6– 4.5×10^8	0.47	0.11	0.36	–	–	–
LPSc 907	5.41×10^5	7.70×10^4–3.10×10^6	0.40	0.09	0.35	–	–	–
Rynaxypyr	40.80	33.55–54.40	3.57	0.70	0.07	–	–	–
Rynaxypyr + LPSc 1067	16.37	11.59–20.05	3.83	0.87	0.96	3.33 (Additive)	48.40 (Synergistic)	15.92 (Synergistic)
Rynaxypyr + LPSc 1082	18.02	13.69–21.71	4.06	0.85	1.08	4.09 (synergistic)	9.42 (synergistic)	41.73 (synergistic)
Rynaxypyr + LPSc 907		NC				1.57 (additive)	16.15 (synergistic)	21.13 (synergistic)
Luphenuron	86.29	72.41–111.75	4.50	0.97	0.39	–	–	–
Luphenuron + LPSc 1067	31.88	23.92–38.87	3.64	0.76	2.91	4.88 (synergistic)	4.82 (synergistic)	19.33 (synergistic)
Luphenuron + LPSc 1082	36.45	NC	3.76	1.56	4.62	6.58 (synergistic)	4.63 (synergistic)	92.78 (synergistic)
Luphenuron + LPSc 907	24.11	16.68–28.93	4.85	1.28	0.05	2.25 (additive)	15.08 (synergistic)	11.36 (synergistic)
Methoxyfenozide	165.09	130.04–296.70	3.83	1.06	0.29	–	–	–
Methoxyfenozide + LPSc 1067	54.12	NC	3.97	1.51	4.10	7.97 (synergistic)	2.41 (additive)	7.65 (synergistic)
Methoxyfenozide + LPSc 1082	64.51	55.00–75.39	5.22	0.87	2.85	11.09 (synergistic)	3.91 (synergistic)	10.30 (synergistic)
Methoxyfenozide + LPSc 907	41.04	32.08–48.55	4.82	1.06	0.19	3.70 (additive)	11.64 (synergistic)	3.05(additive)

In agreement with the results from mortality and MST, the LC_{50} of all chemical insecticides decreased when combined with fungal strains. Moreover, the *M. anisopliae* strain LPSc 907 and rynaxypyr exhibited the lowest LC_{50} 5.4×10^5 conidia/ml and 40.8 ppm respectively, and an additive effect was observed when combined resulting in a decrease of LC_{50}. The combination of chemical substance and LPSc 907 resulted in an additive effect in full doses and synergist effect at 50% and 25% of full dose, except for the combination methoxyfenozide–LPSc907 (at 36 ppm) that produced an additive effect (Table 3). In general, interactions between chemicals and pathogens led to a synergists effect and sometimes to an additive effect; but no antagonism was observed (Table 3). All combinations of chemical insecticides with *B. bassiana* either LPSc 1082 or 1067 resulted in synergism, except for the combination LPSc 1067–rynaxypyr (full field dose) and LPSc 1067-methoxyfenozide 50% which provoked an additive effect. Interactions between chemical insecticide and *B. bassiana* or *M. anisopliae* differed depending on application rate. Although synergism was the most common effect, additivity was more frequently observed at the full field dose than at lower rates (Table 3).

Furthermore, values for the LC_{10} and NEC sublethal toxicological parameters: were 17.9 and 8.0 ppm, respectively, for rynaxypyr; 44.8 and 33.6 ppm for luphenuron; and 76.4 and 58.6 ppm for methoxyfenozide. The LC_{50}/NEC ratio could be adopted as indicative of the safety margin of each particular chemical. This ratio was 4.9, 2.4 and 1.9 for rynaxypyr, luphenuron and methoxyfenozide, respectively being rynaxypyr the more secure substance.

4. Discussion

The entomopathogenic fungi *B. bassiana* and *M. anisopliae* have been widely studied as biocontrol agents of a wide range of insects. The history of their development and safety record has been summarised (Vega, Meyling, Luangsa-ard, & Blackwell, 2012; Zimmermann, 2007a, 2007b). The Orthoptera have been considered targets for use of these fungi for a number of years. The orthopteran-specific *Metarhizium acridum* (Driver & Milner) has been developed for locust control in Africa and Australia. However, a criticism raised in relation to the use of entomopathogenic fungi as biocontrol agents is that the microbial action is too slow (Lomer et al., 2001). The alternative use of chemical insecticides – ideally at low, sublethal levels – in combination with entomopathogenic fungi was developed as an attractive approach to circumvent this difficulty (Bitsadze et al., 2013). A pairing of sublethal concentrations of fungal entomopathogens with insecticides can lead to increase pest mortality as well as reduce the killing time in comparison with either agent alone (Pachamuthu & Kamble, 2000; Paula, Carolino, Paula, & Samuels, 2011).

In this work, we observed that the combinations of insecticides rynaxypyr, luphenuron and methoxyfenozide with *B. bassiana* (strains LPSc 1067, LPSc 1082) and *M. anisopliae* (strain LPSc 907) were more toxic to *D. maculipennis* nymphs than any one of the individual agents used alone. In addition we observed a reduction of MST of *D. maculipennis* nymphs when they were used jointly.

Variations in the effect of the two entomopathogenic fungi *B. bassiana* and *M. anisopliae* on the insect toxicity of chemical insecticides – ranging from antagonistic to neutral to synergistic – have been reported (Bahiense, Fernandes, Angelo, Perinotto, & Bittencourt, 2008; Bitsadze et al., 2013; Mohan, Reddy, Devi, Kongara, & Sharma,

2007; Morales-Rodriguez & Peck, 2009; Purwar & Sachan, 2006; Quintela, Mascarin, Da Silva, Barrigossi, & Martins, 2013; Russell, Ugine, & Hajek, 2010), but few studies have tested the joint action of *B. bassiana* and *M. anisopliae* with chemical insecticides against grasshoppers under field conditions. Different interactions between fungal strains and chemicals on field applications have been observed by some authors. Foster, Reuter, Black, and Britton (1996) observed that a *B. bassiana*–Dimilin™ mixture was more effective than other combinations in field applications against grasshoppers. Delgado, Britton, Onsager, and Swearingen (1999) observed that the effectiveness of the combination diflubenzuron–*B. bassiana* was higher than the effect of the separate components in the control of grasshoppers populations. In our study a synergistic or additive interaction was revealed in all instances when third-instar nymphs of *D. maculipennis* were exposed under semi-field conditions to rynaxypyr, luphenuron and methoxyfenozide simultaneously with entomopathogenic fungi *B. bassiana* and *M. anisopliae*, while antagonistic effects were not observed. On the basis of these observations, the combinations of the tested insecticides with *B. bassiana* (strains LPSc1067, LPSc1082) and *M. anisopliae* (strain LPSc907) were more toxic to *D. maculipennis* nymphs than any one of the individual agents used alone.

Sunlight is known to inactivate *B. bassiana* conidia and grasshoppers are known to thermoregulate (Foster, Reuter, Britton, & Bradley, 2000; Inglis, Johnson, & Goettel, 1997), that is why perhaps we observed that *M. anisopliae* (LPSc 907) strain caused the highest mortality on nymphs of *D. maculipennis* alone (at the three doses tested) or combined with different chemical insecticides. Our results agree with those obtained by Inglis et al. (1997), who observed that the temperature affect the infective capacity of *M. acridum* (presented as *M. flavoviride*; Lomer et al., 2001) and *B. bassiana*, with *M. acridum* more effective at hot temperatures, and *B. bassiana* at cool temperatures. Similar results were observed by Foster et al. (2011) when evaluating the effect of *B. bassiana* and *M. brunneum* against *Anabrus simplex* nymphs (Orthoptera: Tettigoniidae), with mortalities of 28 and 45%, respectively

In conclusion, the comparative effects of rynaxypyr, luphenuron and methoxyfenozide simultaneously with *B. bassiana* and *M. anisopliae* on *D. maculipennis* nymphs indicated that rynaxypyr and *M. anisopliae* (907) were the chemical insecticide and fungal strain that produced higher mortalities. Thus, an IPM strategy for the control of *D. maculipennis* based on the joint action of rynaxypyr–*M. anisopliae* (907), even at a 50% reduction in the recommended field dose, could be a promising alternative for the control of this insect pest.

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Disclosure statement

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References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410. doi:10.1016/S0022-2836(05)80360-2

Bahiense, T. C., Fernandes, E. K. K., Angelo, I. C., Perinotto, W. M. S., & Bittencourt, V. R. E. P. (2008). Performance of *Metarhizium anisopliae* and its combination with deltamethrim against pyrethroid-resistant strain of *Boophilus microplus* in a stall test. *Annals of the New York Academy of Sciences*, *1149*, 242–245. doi:10.1196/annals.1428.031

Bischoff, J. F., Rehner, S. A., & Humber, R. A. (2006). *Metarhizium frigidum* sp. nov.: Acryptic species of M. anisopliae and a member of the *M. flavoviride* complex. *Mycologia*, *98*, 737–745.

Bischoff, J. F., Rehner, S. A., & Humber, R. A. (2009). A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, *101*, 512–530. doi:10.3852/07-202

Bitsadze, N., Jaronski, S., Khasdan, V., Abashidze, E., Abashidze, M., Latchininsky, … Horowitz, A. R. (2013). Joint action of *Beauveria bassiana* and the insect growth regulators diflubenzuron and novaluron, on the migratory locust, *Locusta migratoria*. *Journal of Pest Science*, *86*, 293–300. doi:10.1007/s10340-012-0476-4

Bundey, S., Raymond, S., Dean, P., Roberts, S. K., Dillon, R. J., & Charnley, A. K. (2003). Eicosanoid involvement in the regulation of behavioral fever in the desert locust, *Schistocerca gregaria*. *Archives of Insect Biochemistry Physiology*, *52*, 183–192. doi:10.1002/arch.10081

Carbonell, C. S., Cigliano, M. M., & Lange, C. E. (2006). Acridomorph (Orthoptera) species of Argentina and Uruguay. Publication on Orthopteran diversity. The "Orthopterists Society" and the Museo de la Plata, Argentina. La Plata, CD ROM.

Cigliano, M. M., Pocco, M. E., & Lange, C. E. (2014). Acridoideos (Orthoptera) of agro-economic importance. In S. Roig-Juñent, L. E. Claps, & J. J. Morrone (Eds.), *Arthropod biodiversity of Argentina* (vol. 3, pp. 11–36). San Miguel de Tucuman: INSUE 350 – UNT.

De Wysiecki, M. L., Cigliano, M. M., & Lange, C. E. (1997). Fertility and longevity of adults *Dichroplus elongatus* (Orthoptera: Acrididae) under controlled conditions. *Revista de la Sociedad Entomologica Argentina*, *56*, 101–104.

Delgado, F. X., Britton, J. H., Onsager, J. A., & Swearingen, W. (1999). Field assessment of *Beauveria bassiana* (Balsamo) Vuillemin and potential synergism with Diflubenzuron for control of Savanna grasshoppers complex (Orthoptera) in Mali. *Journal of Invertebrate Pathology*, *73*, 34–39. doi:10.1006/jipa.1998.4804

Elliot, S. L., Blanford, S., & Thomas, M. B. (2002). Host-pathogen interactions in a varying environment: Temperature, behavioural fever and fitness. *Proceedings of the Royal Society of London [Biological Sciences]*, *269*, 1599–1607. doi:10.1098/rspb.2002.2067

El-Sayed, A., & El-Sheikh, Mohamed, M. A. (2011). Comparative effectiveness and field persistence of inset growth regulators on a field strain of the cotton leafworm, *Spodoptera littoralis*, Boisd (Lepidoptera: Noctuidae). *Crop Protection*, *30*, 645–650. doi:10.1016/j.cropro.2011.02.009

Finney, D. J. (1971). *Probit analysis (3rd ed., p. 333). London: Cambridge University Press.*

Fisher, R. A., & Yates, F. (1963). *Statistical tables for biological, agriculture and medicinal research* (6th ed., p. 47). Edinburgh: Oliver and Boyd.

Foster, N. R., Jaronski, S., Reuter, C. K., Lonnie, R. B., Schlothauer, R., Harper, J., & Jech, E. L. (2011). Simulated aerial sprays for field cage evaluation of *Beauveria bassiana* and *Metarhizium brunneum* (Ascomycetes: Hypocreales) against *Anabrus simplex* (Orthoptera: Tettigoniidae) in Montana. *Biocontrol Science and Technology*, *21*, 1331–1350. doi:10.1080/09583157.2011.620080

Foster, R. N., Reuter, K. C., Black, L., & Britton, J. (1996). Evaluation of the fungus *Beauveria bassiana* with selected insecticide stressors for control of unconfined rangeland grasshoppers. *Arthropod Management Tests*, *21*, 280.

Foster, R. N., Reuter, K. C., Britton, J., & Bradley, C. (2000). Lab studies and field trials with the Fungus *Beauveria bassiana* against grasshoppers. In G. L. Cunningham & M. W. Sampson (Eds.), *Grasshopper integrated pest management user handbook* (pp. 1–8). Washington DC: U.S. Dept. of Agriculture, Animal and Plant Health Inspection Service.

Goettel, M. S., & Inglis, G. D. (1997). Fungi: Hyphomycetes. In L. A. Lacey (Ed.), *Manual of techniques in insect pathology (pp. 213–248). San Diego, CA: Academic Press.*

Gonzalez, M., Miglioranza, K. S., Aizpún, J. E., Isla, F. L., & Peña, A. (2010). Assessing pesticide leaching and desorption in soils with different agricultural activities from Argentina (Pampa and Patagonia). *Chemosphere*, *81*, 351–358. doi:10.1016/j.chemosphere.2010.07.021

Inglis, G. D., Johnson, D. L., & Goettel, M. S. (1996). Effect of bait substrate and formulation on infection of grasshopper nymphs by *Beauveria bassiana*. *Biocontrol Science and Technology, 6*, 35–50. doi:10.1080/09583159650039511

Inglis, G. D., Johnson, D. L., & Goettel, M. S. (1997). Effects of temperature and sunlight on mycosis (*Beauveria bassiana*) (Hyphomycetes: Sympodulosporae) of grasshoppers under field conditions. *Environmental Entomology*, *26*, 400–409. doi:10.1093/ee/26.2.400

Jager, T., Heugens, E. H. W., & Kooijman, S. A. L. M., 2006. Making sense of ecotoxicological test results: Towards application of process-based models. *Ecotoxicology*, *15*, 305–314. doi:10.1007/s10646-006-0060-x

Kai, Z. P., Huang, J., Tobe, S. S., & Yang, X. I. (2009). A potential insect growth regulator: Synthesis and bioactivity of an allatostatin mimic. *Peptides*, *30*, 1249–1253. doi:10.1016/j.peptides.2009.03.010

Lacey, L. A., & Brooks, W. M. (1997). Initial handling and diagnosis of diseased insects. In L. A. Lacey & W. M. Brooks (Eds.), *Manual of techniques in insect pathology* (pp. 1–15). San Diego, CA: Academic Press.

Lomer, C. J., Bateman, R. P., Johnson, D. L., Langewald, J., & Thomas, M. (2001). Biological control of locust and grasshoppers. *Annual Review of Entomology*, *44*, 667–702. doi:10.1146/annurev.ento.46.1.667

Mariottini, Y., De Wysiecki, M. L., & Lange, C. E. (2011a). Postembryonic development and food consumption of *Dichroplus elongatus* Giglio-Tos and *Dichroplus maculipennis* (Blanchard) (Orthoptera: Acrididae: Melanoplinae) under laboratory conditions. *Neotropical Entomology*, *40*, 190–196. doi:10.1590/S1519-566X2011000200006

Mariottini, Y., De Wysieki, M. L., & Lange, C. E. (2011b). Longevity and fecundity of *Dichroplus maculipennis* (Orthoptera, Acrididae) at non-outbreaking and outbreaking situations. *Revista Brasileira de Entomologia*, *55*, 435–438. doi:10.1590/S0085-56262011005000024

Mohan, M. C., Reddy, N. P., Devi, U. K., Kongara, R., & Sharma, H. C. (2007). Growth and insect assays of *Beauveria bassiana* with neem to test their compatibility and synergism. *Biocontrol Science and Technology*, *17*, 1059–1069. doi:10.1080/09583150701714551

Morales-Rodriguez, A., & Peck, D. C. (2009). Synergies between biological and neonicotinoid insecticides for the curative control of the white grubs *Amphimallon majale and Popillia japonica*. *Biological Control*, *51*, 69–180. doi:10.1016/j.biocontrol.2009.06.008

Nishimatsu, T., & Jackson, J. J. (1998). Interaction of insecticides, entomopathogenic nematodes, and larvae of the western corn rootworm (Coleoptera: Chrysomelidae). *Journal of Economy Entomology*, *91*, 410–418. doi:10.1093/jee/91.2.410

Nussenbaum, A. L., & Lecuona, R. E. (2012). Selection of *Beauveria bassiana* sensu lato and *Metarhizium anisopliae* sensu lato isolates as microbial control agents against the boll weevil (Anthonomus grandis) in Argentina. *Journal of Invertebrate Pathology*, *110*, 1–7. doi:10.1016/j.jip.2012.01.010

Pachamuthu, P., & Kamble, S. T. (2000). In vivo study on combined toxicity of *Metarhizium anisopliae* (Dictyoptera: Blattellidae). *Journal of Economic Entomology*, *93*, 60–70. doi:10.1603/0022-0493-93.1.60

Paula, A. R., Carolino, A. T., Paula, C. O., & Samuels, R. I. (2011). The combination of the entomopathogenic fungus *Metarhizium anisopliae* with the insecticide imidacloprid increases virulence against the dengue vector *Aedes aegypti* (Diptera: Culicidae). *Parasites & Vectors, 4*, 1–8.

Pelizza, S. A., Eliades, L. A., Saparrat, M. C. N., Cabello, M. N., Scorsetti, A. C., & Lange, C. E. (2012). Screening of Argentine native fungal strains for biocontrol of the grasshopper *Tropidacris collaris*: Relationship between fungal pathogenicity and chitinolytic enzyme activity. *World Journal of Microbiology and Biotechnology*, *28*, 1359–1366. doi:10.1007/s11274-011-0935-8

Pelizza, S. A., Eliades, L. A., Scorsetti, A. C., Cabello, M. N., & Lange, C. E. (2012). Entomopathogenic fungi from Argentina for the control of *Schistocerca cancellata* (Orthoptera: Acrididae) nymphs: Fungal pathogenicity and enzyme activity. *Biocontrol Science and Technology*, *22*, 1119–1129. doi:10.1080/09583157.2012.713910

Péry, A. R., Flammarion, P., Vollat, B., Bedaux, J. J., Kooijman, S. A., & Garric, J. (2002). Using a biology-based model (DEBtox) to analyze bioassays in ecotoxicology: Opportunities and recommendations. *Environmental Toxicology and Chemistry*, *21*, 459–465.

Purwar, J. P.,& Sachan, G. C. (2006). Synergistic effect of entomogenous fungi on some insecticides against Bihar hairy caterpillar *Spilarctia obliqua* (Lepidoptera: Arctiidae). *Microbiological Research*, *161*, 38–42. doi:10.1016/j.micres.2005.04.006

Quintela, E. D., Mascarin, G. M., Da Silva, R. A., Barrigossi, J. A. F., & Martins, J. F. (2013). Enhanced susceptibility of *Tibraca limbativentris* (Heteroptera: Pentatomidae) to *Metarhizium anisopliae* with sublethal doses of chemical insecticides. *Biological Control*, *66*, 56–64. doi:10.1016/j.biocontrol.2013.03.018

Rehner, S. A., & Buckley, E. (2005). A Beauveria phylogeny inferred from nuclear ITS and EF1- sequences: evidence for cryptic diversification and links to Cordyceps teleomorphs. *Mycologia*, *97*, 84–98. doi:10.3852/mycologia.97.1.84

Russell, C. W., Ugine, T. A., & Hajek, A. E. (2010). Interactions between imidacloprid and *Metarhizium brunneum* on adult Asian longhorned beetles (*Anoplophora glabripennis* (Motschulsky)) (Coleoptera: Cerambycidae). *Journal of Invertebrate Pathology*, *105*, 305–311. doi:10.1016/j.jip.2010.08.009

SAS/STAT® Version 8. (2001). *The GLM procedure*. Cary, NC: SAS Institute, SAS Campus Drive.

Stenglein, S., & Balatti, P. (2006). Genetic diversity of *Phaeoisariopsis griseola* in Argentina as revealed by pathogenic and molecular markers. *Physiological and Molecular Plant Pathology*, *68*, 158–167

Vega, F. E., Meyling, N. V., Luangsa-ard, J. J., & Blackwell, M. (2012). Fungal entomopathogens. In F. E. Vega & H. K. Kaya (Eds.), *Insect pathology* (pp. 171–220). London: Elsevier.

White, T. J., Bruns, T., Lee, S., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols: A guide to methods and applications* (pp. 315–322). San Diego, CA: Academic Press.

Xlstat Life Software, 2013. France: Addinsoft SARL Paris. Retrieved from http://www.xlstat.com

Zimmermann, G. (2007a). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology*, *17*, 553–596. doi:10.1080/09583150701309006

Zimmermann, G. (2007b). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology*, *17*, 879–920. doi:10.1080/09583150701593963