Compatibility between entomopathogenic fungi and biorational insecticides in toxicity against *Ronderosia bergi* under laboratory conditions

Sebastian A. Pelizza · A. C. Scorsetti ·
M. N. Fogel · S. G. Pacheco-Marino ·
S. A. Stenglein · M. N. Cabello · C. E. Lange

Abstract Our aim was to evaluate the efficacy of combinations between two biorational insecticides (luphenuron, methoxyfenozide), a new synthetic chemical pesticide (rynaxypyr), and three entomopathogenic fungi strains (*Beauveria bassiana* LPSc 1067, LPSc1082), and *Metarhizium anisopliae* (LPSc 907) in the biocontrol of the pest grasshopper *Ronderosia bergi* (Stål) under laboratory conditions. The insecticides were tested at three concentrations: the average concentration recommended for application in the field (100 %) and 50 % and finally 25 % of that level. The fungal strains used were adjusted to 1×10^8, 1×10^6, and 1×10^4 conidia ml^{-1}. The combinations of those insecticides with *B. bassiana* (LPSc 1067, LPSc 1082) and *M. anisopliae* (LPSc 907) caused higher mortality to *R. bergi* nymphs than any of the individual agents used alone. The three insecticides tested did not affect the isolates of the two species of entomopathogenic fungi employed. In conclusion, the use of these biorational insecticides in an IPM program aimed at control of the grasshopper *R. bergi* could be of value.

Keywords Grasshoppers · Interactions · *Metarhizium anisopliae* · *Beauveria bassiana* · Pesticides · Orthoptera: Acrididae

Introduction

As in other temperate grasslands of the world, throughout the Argentine Pampas and parts of Patagonia grasshoppers are among the most economically

S. A. Pelizza (✉) · M. N. Fogel · C. E. Lange
Centro de Estudios Parasitológicos y de Vectores
(CEPAVE), CCT La Plata-CONICET-UNLP, Boulevard
120 s/n e/ 61 y 62, 1900 La Plata, Argentina
e-mail: pelizza@cepave.edu.ar

S. A. Pelizza · A. C. Scorsetti · M. N. Cabello
Instituto de Botánica Carlos Spegazzini (FCNyM-UNLP),
Calle 53 # 477, 1900 La Plata, Argentina

S. G. Pacheco-Marino
Instituto de Genética Veterinaria Ingeniero Fernando Noel
Dulout (IGEVET), CCT La Plata-CONICET, La Plata,
Argentina

S. A. Stenglein
Laboratorio de Biología Funcional Y Biotecnología
(BIOLAB)-CICBA-INBIOTEC, Facultad de Agronomía
de Azul, UNCPBA, Republica de Italia # 780, 7300 Azul,
Argentina

M. N. Cabello · C. E. Lange
Comisión de Investigaciones Científicas (CIC) de la
Provincia de Buenos Aires, La Plata, Argentina

significant invertebrate native herbivores (Mariottini et al. 2011). *Ronderosia bergi* (Orthoptera: Acrididae) (Stål), the melanopline grasshopper selected for this study is characterized by no obligatory embryonic diapause. This is one of 18 species found in natural grasslands that affects certain crops in areas of central and northern Argentina and is thus considered economically relevant (Mariottini et al. 2010). To date, chemical insecticides are the only available option for grasshopper control in Argentina, but the use of such chemical agents is of serious environmental concern (Gonzalez et al. 2010). A recognition of the drawbacks associated with insecticide use—such as non-target effects, environmental pollution, and high economic costs—have prompted the development of alternative control strategies and environmentally less harmful chemicals such as insect-growth regulators. Those types of pesticides have a relatively minor detrimental effect on the environment and the other inhabitants therein (Horowitz and Ishaaya 2004).

The different problems caused by the conventional chemical insecticides have led to the application of alternative methods such as the use of entomopathogens. Among these, control agents based on fungi have attracted much attention as biological pesticides. *Beauveria bassiana* (Bals.-Criv) Vuillemin, and *Metarhizium anisopliae* (Metschn.) Sorokin (Ascomycota: Hypocreales) are two of the most common species of entomopathogenic fungi that have been investigated, with both offering promise in the microbial control of certain crop pests (Jaronski 2010).

A criticism raised in relation to the use of entomopathogenic fungi as biocontrol agents, however, 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 has developed as an attractive approach to circumvent this difficulty (Bitsadze et al. 2013). Indeed, even though insects killed by entomopathogenic fungi often take longer to die than those treated with chemical pesticides, the damage to crops decrease during the disease incubation period since the infected insects tend to eat less than healthy ones, modify their survival and their reproductive potential (Moore et al. 1992; Pelizza et al. 2013).

Previous investigations have demonstrated that sublethal doses of insecticides can increase stress, compromise the immune system, and alter insect behavior so as to lead to an improved effectiveness of

the fungal pathogen (Quintela et al. 2013). The utilization of appropriately selected insecticides in association with entomopathogenic fungi can increase the efficiency of pest control, allowing a consequent reduction in the amount of the chemical that is applied along with a minimization of environmental contamination and the expression of pest resistance (Oliveira et al. 2003; Paula et al. 2011). Earlier reports have suggested that Dimilin[TM] (Uniroyal Chemical Co., Inc), a chitin-synthesis inhibitor containing the active ingredient diflubenzuron, makes infection by hypocrealean entomopathogens faster and easier. Consequently, Hassan and Charnley (1989) found that the cuticle of *Manduca sexta* treated with that agent exhibited a diminished resistance to invasion by the hyphae of *M. anisopliae*. In vitro assays performed by Reuter et al. (1996) furthermore demonstrated that an admixture of diflubenzuron and *B. bassiana* increased the percent mortality of a North-American grasshopper species. Similar results were reported by Foster et al. (1996) when a natural population was exposed to the same mixture under field conditions.

Since grasshoppers in general are susceptible to infection by entomopathogenic fungi, the compatibility of chemical insecticides with the fungi *B. bassiana* and *M. anisopliae* in the control of *R. bergi* is a consideration of major relevance in the development of an integrated pest-management (IPM) strategy for the protection of vulnerable crops (Horowitz and Ishaaya 2004). In this article, we evaluated the interactions between three insecticides of which two are considered biorational and three strains of entomopathogenic fungi, under laboratory conditions in order to determine the possible usefulness of combinations of these agents against the pest grasshopper *R. bergi*.

Materials and methods

Insect colony

Adult males and females of *R. bergi* were collected with insect nets in grasslands at Pereyra Iraola Provincial Park (34°86′56″S, 58°06′68″W), Berazategui county, Buenos Aires province Argentina. Once in the laboratory, the grasshoppers were maintained following the general procedures described by Henry (1985). Individuals of both sexes were placed in wire-screened, aluminum cages (20 × 20 × 30 cm) in a

rearing room under the controlled conditions used worldwide (30 °C, 14:10 L:D period, 40 % RH; Mariottini et al. 2010). The grasshoppers were fed daily with thoroughly washed fresh leaves of a variety of grasses, lettuce, cabbage, and wheat-bran flakes. Each cage was provided with substrates for egg-pod laying that consisted of plastic containers (10 cm deep) filled with sterilized sand. Thermoregulation and mating was stimulated by the use of 75-W bulbs suspended 15 cm above each cage. The grasshoppers were maintained until death, at which point they were immediately examined upon dissection (Lange 1996) or else incubated in humid chambers (Lacey and Brooks 1997) to check for their health status. The nymphs emerging from the resulting egg-pods were used in the laboratory assays.

Insecticides and fungal strains

The synthetic chemical pesticide Coragen[TM] [rynaxy-pyr 20 % (w/v), DuPont S.A., Argentina] and two biorational chemical insecticides Match[TM] [luphenu-ron 5 % (w/v), Sygenta S.A., Argentina] and Intrepid[TM] [methoxyfenozide 24 % (w/v), Dow AgroSciences S.A., Argentina] were used in these experiments. Rynaxypyr acts as an activator of the ryanodine receptors of insects to undermine 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 to accelerate molting. The insecticides were tested at three concentrations: the average concentration recommended for application in the field on soybean (100 %), 50 % of this level, and finally 25 % to give levels of 60, 30, and 15 ppm (mg a.i. l^{-1}) for rynaxypyr; 100, 50, and 25 ppm (mg a.i. l^{-1}) for luphenuron; and 144, 72, and 36 ppm (mg a.i. l^{-1}) for methoxyfenozide (CASAFE 2013).

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 lyophiliza-tion. Morphological species identification was corrob-orated 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/ITS4 (Rehner and Buckley 2005) and the translation elongation factor 1-alpha (EF intron region) of *M. anisopliae* using primer pairs EF1T/EF2T (Bischoff et al. 2009). Fragments simi-larities with previously published sequence data were examined with BLASTn (Altschul et al. 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, 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 et al. 2012a, b). Conidia of the fungal strains were obtained from cultures on potato-dextrose-agar medium (Britania S.A., Buenos Aires, Argentina) after incubation for ten days at 25 °C in the dark. Afterward, the conidia were harvested with disposable cell scrapers (Fisher-brand[TM]) and placed in test tubes containing 0.01 % (v/v) polyoxyethylene sorbitan monolaurate (Tween 80[TM]; 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^{-1} 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 Lane et al. (1988). 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 %.

Compatibility in vitro of fungal isolates
with chemical insecticides

In vitro compatibility between isolates of *B. bassiana* (LPSc 1067, LPSc 1082) or *M. anisopliae* (LPSc 907) and the three insecticides commonly used for pest control was studied at the three insecticide concentra-tions cited above along with the highest concentration of each of the fungal strains (i.e., 1×10^8 conidia ml^{-1}). An admixture of 1 ml of fungal suspension plus 1 ml of each chemical concentration was performed to give a final volume of 2 ml. After 1 h, 0.5 ml of each mixture was spread over three Petri dishes containing water-agar medium. The dishes were then incubated at 25 °C for a 12-h photoperiod and the percentage of germinated conidia quantified 24 h later. A control without the addition of chemical insecticide was made

for each fungal isolate. The data were analysed using the Wald statistics (Generalized Linear Model) with the Genmod procedure with the software SAS/STAT® Version 8 (2001), with a binomial error distribution and a logit link function. Differences between treatments were detected using a posteriori Fisher LSD test with Statistica 7 software.

Experimental inoculations

Experimental inoculations *per os* were conducted on third-instar nymphs of *R. bergi* in order to determine the percent mortality at each concentration of the insecticide and different densities of the three fungal strains. We chose this inoculation technique on the basis of its recommendation by the manufacturer of all three of the chemical insecticides. Twenty-four hours before the beginning of the experiment, the nymphs were placed individually in 20 ml foam-plugged glass vials and left without food. Lettuce disks (5 mm in diameter) submerged in desired concentrations of fungi and insecticides were given to treated groups according to Inglis et al. (1996). Control groups were fed with lettuce disks submerged in 1 ml of 0.01 % (v/v) Tween 80[TM] only. Under each experimental condition ten third-instar nymphs were processed in triplicate for both the treated and control insects. Immediately after feeding, the treated and control nymphs were placed in acetate tubes (50 × 9-cm), in groups of ten individuals, and fed with lettuce, cabbage, and wheat bran. Both the treated and control insects were maintained at 30 °C, 60 % RH, and a 14:10 L:D photoperiod. The cumulative mortality was recorded daily for ten days. Dead grasshoppers with no external mycelium were surface-sterilized 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, for 35 days to allow fungal development. Mycosis was confirmed by microscopical examination of the dead grasshoppers. When the mortality in the treated grasshoppers at different concentrations (1×10^8, 1×10^6, and 1×10^4 conidia ml^{-1}) of the three fungal strains and different chemical compounds (60, 30, and 15 ppm for rynaxypyr; 100, 50, and 25 ppm for

luphenuron; and 144, 72, and 36 ppm for methoxyfenozide) was 50 % or higher, the median survival time (MST) was calculated based on Kaplan-Meier estimate of the survival distribution function (Xlstat life software 2014). Pairwise comparisons between survival curves were made by log-rank test. Moreover, when the mortality was 50 % or higher in the grasshoppers treated with the different chemical compounds alone, the following lethal and sublethal-toxicity parameters were calculated (DEBtox v. 2.0.1 program; Péry et al. 2002; Jager et al. 2006): the median lethal concentration (LC$_{50}$), the concentration lethal to 10 % of the exposed individuals (LC$_{10}$), the non-effect concentration (NEC). Significant differences between the percent mortalities after a ten-day exposure to the different treatments were analysed and a posteriori test of linear contrast led us to measure the significance among combinations using the Wald statistics (Generalized Linear Model) with the Genmod procedure with the software SAS/STAT® Version 8 (2001), with a binomial error distribution and a logit link function.

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

The interaction of *B. bassiana* (LPSc 1067, LPSc 1082) and *M. anisopliae* (LPSc 907) with the insecticides rynaxypyr, luphenuron, and methoxyfenozide against *R. bergi* grasshoppers 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 above mentioned concentration for each fungal strain and chemical insecticide evaluated). Suspensions were homogenized by vortexing for 2 min. A lettuce disk (diameter, 5 mm) was submerged in each mixture. The experimental design and inoculation techniques was performed as indicated in the previous section. The cumulative mortality was recorded daily for ten days and mycosis confirmed by microscopical examination of the dead grasshoppers. When mortality was 50 % or higher, the toxicologic effect of rynaxypyr, luphenuron, and methoxyfenozide on third-instar *R. bergi* nymphs was 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 (SD). 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 $P_e = P_o + (1 - P_o)(P_1) + (1 - P_o)(1 - P_1)(P_2)$, 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. 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 $P_c > P_e$.

Results

We observed significant differences (Table 1) in the in vitro viability of the fungal conidia in the presence of the three chemical insecticides, however the percent of germination was not affected negatively, suggesting compatibility between these two different control agents (Table 2). When we compared the percent mortality of third-instar *R. bergi* nymphs registered after a ten-day exposure, significant differences were found with the chemical insecticides at their maximum recommended field concentration (100 %) and at the reduced concentration (25 %) (Table 3). In addition, the percent mortality obtained after exposure of the nymphs to the fungal strain alone was higher than those with the insecticides alone at those same maximum concentrations ($\chi^2 = 140.63$, $df = 2$, $P < 0.0001$). Moreover, the combined treatment with strains LPSc 907, LPSc 1082 or LPSc 1067 and the compounds produced 100 % mortality in the nymphs, except for the combination of luphenuron with strain LPSc 1067, where the mortality was 80 % (Fig. 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 *R. bergi* nymphs after exposure to the two agents in combination compared to treatments with those compounds or the fungi alone ($\chi^2 = 160.42$, $df = 2$, $P < 0.0001$), except for the combination luphenuron-LPSc 1067 (66.6 % ± 0.7) that was lower than fungus control (73.3 % ± 0.82) ($\chi^2 = 45.97$, $df = 2$, $P < 0.0001$). The percent mortalities caused by the all fungal strains (*M. anisopliae* LPSc 907, *B. bassiana* LPSc 1067 and LPSc 1082) were higher than the values recorded for any of the chemicals alone and significant differences between fungal strains were found (Fig. 1b; Table 3). At 25 % of maximum concentrations we observed significant differences in the percent mortalities of fungal strains, chemicals insecticide and the combination between both (Table 3). The chemicals and fungal strains showed additive effect when combined, except the combination rynaxypir-LPSc 1067 and rynaxypyr-LPSc 907 that showed synergism (Table 4). Also, we observed that the combination rynaxypyr-LPSc 907 produced the highest mortality. In addition, LPSc 1067 and LPSc 1082 gave rise to their higher percent mortalities in combination with rynaxypyr ($\chi^2 = 128.62$, $df = 2$, $P < 0.0001$) and luphenuron ($\chi^2 = 49.93$, $df = 2$, $P < 0.0001$) respectively (Fig. 1c).

Table 4 shows the toxicological parameter (LC_{50}) determined after a ten-day exposure to each pesticide alone and in combination with the various fungal strains. Rynaxypyr alone ($LC_{50} = 39.5$ ppm) was the chemical with the highest value, whereas methoxyfenozide showed the lowest ($LC_{50} = 171$ ppm). In all instances a lower LC_{50} was obtained when combining any of the three pesticides with the different fungal isolates (Table 4). At maximum field concentrations, we observed an additive effect in all combinations. At 50 % concentration we observed synergism in the combinations rynaxypir-LPSc1082-LPSc907,

Table 1 Results of the Generalized Linear Model on the effects of the chemical insecticides rynaxypyr, luphenuron and methoxyfenozide on the percent germination of *Beauveria bassiana* (strains LPSc 1067, LPSc 1082) and *Metarhizium anisopliae* (strain LPSc 907) conidia

Dose	100 %			50 %			25 %		
	df	χ^2	*P* value	df	χ^2	*P* value	df	χ^2	*P* value
Insecticides	3	156.08	<0.0001	3	101.93	<0.0001	3	143.84	<0.0001
Strains	2	222.98	<0.0001	2	316.87	<0.0001	2	344.98	<0.0001
Insecticides × strains	6	163.32	<0.0001	6	24.37	0.0004	6	172.84	<0.0001

Table 2 Mean percent ± SD conidial viability of strains *Beauveria bassiana* LPSc 1067, LPSc 1082 and *Metarhizium anisopliae* LPSc 907 both alone and in combination at maximum field concentration (100 %) and at concentrations of the chemical agents reduced to 50 and 25 %

Insecticides concentrations (%)	Rynaxypyr			Luphenuron			Methoxyfenozide		
	LPSc 907	LPSc 1082	LPSc 1067	LPSc 907	LPSc 1082	LPSc 1067	LPSc 907	LPSc 1082	LPSc 1067
0	98 ± 1.6a	85.2 ± 3.6a	97.1 ± 2.2a	98 ± 1.6a	85.2 ± 3.6a	97.1 ± 2.2a	98 ± 1.6a	85.2 ± 3.6a	97.1 ± 2.2a
25	96.8 ± 1.5a	89.1 ± 4.4ab	97.7 ± 1.7a	98.3 ± 2.1ab	79.2 ± 6a	98.4 ± 1.3a	79.5 ± 4.6a	85.1 ± 4.2a	94.5 ± 2.7a
50	98.8 ± 1.2a	90.5 ± 3.8ab	99.4 ± 0.5a	91.4 ± 4.6a	79.5 ± 4.4a	94.3 ± 2.1bc	93.8 ± 3.3a	83.9 ± 2.3a	96.8 ± 0.8a
100	99.1 ± 1.3a	90.8 ± 3.6b	99.7 ± 0.3a	79.3 ± 4b	84.8 ± 7.8a	92.7 ± 2.3c	97.1 ± 2.4a	81.7 ± 3.19a	96.4 ± 1.4a

Different letters denote significant differences between treatments in the same column according to the Fisher LSD test ($P < 0.05$)

luphenuron-LPSc1082-LPSc907, and methoxyfenozide-LPSc 907, while in the other combinations we observed an additive effect. Finally, at 25 % concentration of the pesticides we observed only a synergism effect with the combinations rynaxypyr-LPSc907–LPSc1067, while in all other combinations the effect was additive (Table 4). Furthermore, we found similar values for the LC_{10} and NEC sublethal toxicological parameters: those values were 27.5 and 25.1 ppm, respectively, for rynaxypyr; 100 and 100 ppm for luphenuron; and 58.0 and 36.0 ppm for methoxyfenozide.

The MST calculated for the strains, the chemicals and their combinations at 100 % concentration showed that the most virulent combinations were luphenuron-LPSc 1082 and rynaxypyr-LPSc 1067 with MST of 1.8 and 1.9 days respectively, which were significantly different than the rest of the treatments (log-rank test, $P < 0.05$). On the other hand, the highest MST were observed in the strain LPSc1082 (5.4 days) and in combination with methoxyfenozide (5.7 days), being significantly different than the rest of the treatments (log-rank test, $P < 0.05$). At 50 % concentration, the lowest MST was 3.6 days, observed in the treatments rynaxypyr-LPSc 907 and luphenuron-LPSc 1082, which were significantly different than the other treatments (log-rank test, $P < 0.05$). As observed for the 100 % concentration, the treatments LPSc1082 and in combination with methoxyfenozide have MST significantly higher than the rest of the treatments (log-rank test, $P < 0.05$). At 25 % concentration the MST were in general high and similar among treatments, only the combination rynaxypyr-LPSc 907 have a significantly lower MST compared with the rest of the treatments (log-rank test, $P < 0.05$) (Table 5).

Discussion

Combination of sublethal concentrations of chemical insecticides and entomopathogenic fungi can cause increased stress, immunocompromise, and a consequent alteration in insect physiology and behavior, thus leading to an improved of performance in an insect-control program that include a biological component (Boucias et al. 1996). A pairing of sublethal concentrations of insecticides with fungal entomopathogens can increase pest mortality as well as reduce the killing time in comparison with either agent alone

Table 3 Results of the Generalized Linear Model on the percent mortality of third-instar *Ronderosia bergi* nymphs after ten-day exposure at different concentrations of biorational pesticides alone and in combination with the *Beauveria bassiana* LPSc 1067, LPSc 1082, and *Metarhizium anisopliae* LPSc 907 strains

Dose	100 %			50 %			25 %		
	df	χ^2	*P* value	df	χ^2	*P* value	df	χ^2	*P* value
Insecticides	3	275.82	<0.0001	3	0.00	1	3	40.28	<0.0001
Strains	2	934.18	<0.0001	2	10872.7	<0.0001	2	18.93	0.0003
Insecticides × strains	6	137.32	<0.0001	6	13299.0	<0.0001	6	43.59	<0.0001

(Paula et al. 2011). Although studies on the joint action of chemical insecticides and entomopathogenic fungi have been carried out on the binary management of the locust (Bitsadze et al. 2013), similar investigations on other grasshopper pests, such as *R. bergi*, have been scant.

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 (Quintela et al. 2013; Bitsadze et al. 2013). In the present work, although the three different insecticides utilized exhibited varying effects (synergistic or additive) with respect to toxicity against *R. bergi* when combined with strains from either *B. bassiana* or *M. anisopliae,* we never observed an antagonism effect between them. On the basis of these observations, the combinations of those insecticides with *B. bassiana* (LPSc 1067, LPSc 1082) and *M. anisopliae* (LPSc 907) were more toxic to *R. bergi* nymphs than either of the individual agents used alone.

The three insecticides tested (rynaxypyr, luphenuron, methoxyfenozide) did not affect the isolates of the two species of entomopathogenic fungi employed. Recent studies have indicated that various oil-based surfactants may impact on the viability and virulence of fungal entomopathogens in several ways (Mascarin et al. 2014). Anderson and Roberts (1983) pointed out that the formulation of an insecticide is just as significant as the active ingredient and in certain instances may increase the number of entomopathogenic-fungal colonies. Moino and Alves (1998) suggested two possible explanations for these results: First, the fungi—as a physiologic mechanism of resistance—could metabolize the insecticides and liberate compounds that can be used by them as secondary nutrients. Second, within a toxic environment, the fungus could respond with an enhanced reproductive effort in the form of an increased conidia production. Another possible explanation is that toxicologically inert substances present in the insecticide formulations could be used directly as nutrients so as to increase the vegetative growth and conidia production of the pathogen (Neves et al. 2001).

Compatibility studies in vitro between insecticides and entomopathogenic fungi have the advantage of exposing the pathogen to the maximum action of the compounds, a condition that often does not occur under field conditions. Thus, compatibility in vitro with an insecticide is strong evidence in favour of the utility of that combination under field conditions (Alves et al. 1998). Oliveira et al. (2003) reported that formulations of insecticides based on triazophos, chlorpyrifos, and endosulfan inhibited the viability of *B. bassiana* conidia 100 % at the three concentrations used. Moreover, they ascertained that conidia germination in the presence of commercial pesticides in vitro was one of the most informative indicators of compatibility in the field.

Different interactions between fungal strains and chemicals on field applications have been observed by different authors. Previous field applications against grasshoppers in the United States (Foster et al. 1996) demonstrated that a *B. bassiana*–Dimilin[TM] combination was more effective than the combination *B. bassiana* with other chemical agents. Delgado et al. (1999) observed in populations of grasshoppers in Mali treated with a mixture of *B. bassiana* and Dimilin[TM] that the decrease in the insects populations continued until the end of the monitoring effort. Thus, when an IPM strategy is devised, the compatibility of products sprayed on the crop must be taken into account in order to avoid the use of the most toxic compounds (van Driesche et al. 2007).

In general, the use of the insecticides rynaxypyr, luphenuron, and methoxyfenozide in the formulations

Fig. 1 Mean percent ± SD mortality of *Ronderosia bergi* 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 %
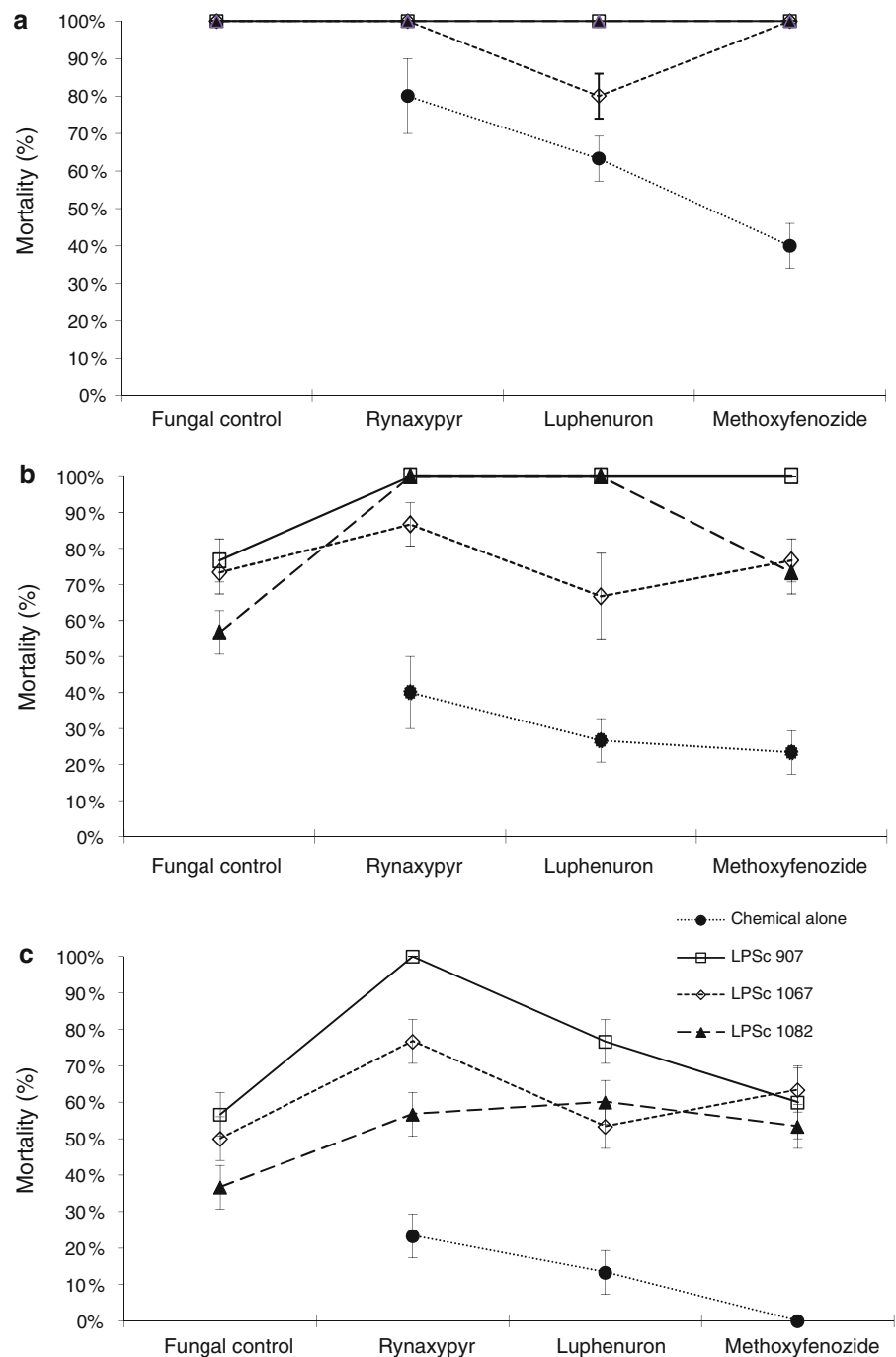
and at the concentrations tested here failed to have an adverse effect on the conidial viability of *B. bassiana* (LPSc 1067, LPSc 1082) and *M. anisopliae* (LPSc 907). Furthermore, when comparing the mortality caused by these three insecticides alone and in combination with different fungal isolates, for each of those compounds we observed a similar percent mortality with *R. bergi* nymphs, even at a 50 % reduction in the recommended field dose. Our results revealed that the different combinations between chemical insecticides and fungal strains showed synergistic and additive effects but not antagonism.

Table 4 Median lethal concentrations (LC50) of rynaxypyr, luphenuron, and methoxyfenozide alone and in combination with the entomopathogenic fungal strains LPSc 907, LPSc 1067, and LPSc 1082 against *Ronderosia bergi* after a ten-day exposure

	Field dose (100 %)			50 %			25 %		
	LC_{50}	SD	χ^2 (response)	LC_{50}	SD	χ^2 (response)	LC_{50}	SD	χ^2 (response)
Rynaxypyr	39.5	3	–	70.4	48.8	–	75.7	–	–
Rynaxypyr + 1067	12.4	1.82	0.00 (additive)	9.74	3.48	0.03 (additive)	8.09	1.81	3.85 (synergistic)
Rynaxypyr + 1082	11.7	2.26	0.00 (additive)	12.6	2.63	10.56 (synergistic)	14.7	6.55	0.37 (additive)
Rynaxypyr + 907	5.46	1.18	0.00 (additive)	4.97	1.03	4.87 (synergistic)	4.72	1.4	14.97 (synergistic)
Luphenuron	100	3.96	–	203	243	–	25	0.846	–
Luphenuron + 1067	32.1	4.6	1.20 (additive)	28.3	3.74	3.32 (additive)	23.6	3.65	0.08 (additive)
Luphenuron + 1082	21.8	1.96	0.00 (additive)	21.6	2.48	14.05 (synergistic)	25	0.0092	2.72 (additive)
Luphenuron + 907	14.1	3.59	0.00 (additive)	14.4	4.56	9.16 (synergistic)	20.1	12.5	2.60 (additive)
Methoxyfenozide	171	32.4	–	72	0.98	–	–	–	–
Methoxyfenozide + 1067	33.1	5.16	0.00 (additive)	33.8	5.93	0.15 (additive)	36	0.0086	3.37 (additive)
Methoxyfenozide + 1082	40.4	5.86	0.00 (additive)	37.5	5.97	0.58 (additive)	34.1	6.75	3.61 (additive)
Methoxyfenozide + 907	22.2	5.63	0.00 (additive)	25.2	6.41	6.58 (synergistic)	29.3	13.6	0.14 (additive)

The number of dead grasshoppers observed, and the number of dead grashoppers 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 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 $P_c > P_e$

Table 5 Median survival time in days (mean ± SD) at different concentrations of fungi and pesticides alone and in combination with the *Beauveria bassiana* LPSc 1067, LPSc 1082 and *Metarhizium anisopliae* LPSc 907strains

Treatments	Dose		
	100 %	50 %	25 %
LPSc 907	3.45 ± 0.34ab	6.5 ± 0.61ag	8 ± 0.37a
LPSc 907 + Rynaxypyr	2.7 ± 0.26a	3.6 ± 0.34b	5.6 ± 0.52b
LPSc 907 + Luphenuron	2.9 ± 0.30a	4.8 ± 0.45cdh	6.5 ± 0.51bc
LPSc 907 + Methoxyfenozide	3.6 ± 0.37ab	4.3 ± 0.44ce	6.9 ± 0.47ac
LPSc 1082	5.4 ± 0.53c	8 ± 0.46a	–
LPSc 1082 + Rynaxypyr	3.2 ± 0.31ad	5.5 ± 0.55df	7.2 ± 0.57ac
LPSc 1082 + Luphenuron	1.8 ± 0.13e	3.6 ± 0.36be	6.6 ± 0.48ac
LPSc 1082 + Methoxyfenozide	5.7 ± 0.59c	7.4 ± 0.5a	8.4 ± 0.46a
LPSc 1067	4 ± 0.32bdfg	6.6 ± 0.58af	–
LPSc 1067 + Rynaxypyr	1.9 ± 0.15e	5.5 ± 0.6fgh	7.3 ± 0.49ac
LPSc 1067 + Luphenuron	3 ± 0.27af	6.3 ± 0.45af	8.4 ± 0.4a
LPSc 1067 + Methoxyfenozide	3.5 ± 0.39af	5.4 ± 0.53gdf	7.7 ± 0.47ac
Rynaxypyr	4 ± 0.4bdfg	–	–
Luphenuron	5 ± 0.4cg	–	–
Methoxifenozide	–	–	–

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

Further field studies with these chemical insecticides and entomopathogenic fungi applied as a mixture can provide additional information on the insect toxicity by these combinations as well as their effectiveness against *R. bergi* under natural conditions. Such biorational insecticides could conceivably reduce concentrations of chemical compounds to the benefit of the farmer and the environment.

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Sebastian A. Pelizza has studied the effects of entomopathogenic fungi on grasshoppers and locusts (Orthoptera: Acrididae) with the purpose of an integrated pest management.

A. C. Scorsetti has investigated the entomopathogenic fungi on horticultural pest and its interaction with natural enemies (Coleoptera: Coccinellidae).

M. N. Fogel has studied the eco-toxicology of chemical insecticides on natural enemies of insect pest.

S. G. Pacheco-Marino has studied the toxicology of pathogenic fungi on fishes.

S. A. Stenglein is expert on physiological and molecular basis of the *Fusarium–Poaceae* interaction.

M. N. Cabello is specialist on taxonomy of Imperfecti fungi and mycorrhizas.

C. E. Lange is specialist on biology, ecology and control of grasshoppers and locusts (Orthoptera: Acrididae).