Within-host interactions of *Metarhizium rileyi* strains and nucleopolyhedroviruses in *Spodoptera frugiperda* and *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)

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**ABSTRACT**

Members of the family Baculoviridae have been quite successfully used as biocontrol agents against some lepidopterans. Likewise, a number of fungi are important natural enemies of these pests. An interesting approach to increase control efficacy could be the combination of a given nucleopolyhedrovirus (NPV) and a fungus, since they possess distinct modes of action. As a first step towards this goal, we assessed the interaction between NPV (either AgMNPV-79 or SfMNPV-6nd) and the entomopathogenic fungus *Metarhizium rileyi* (either CG1153 or CG381), using *Anticarsia gemmatalis* and *Spodoptera frugiperda* as hosts. In sequential applications of these pathogens, per os inoculation of an NPV (leaf discs with $2.5 \times 10^7$ occlusion bodies) either two days before or two days post-spraying of its counterpart fungal strain ($5 \times 10^3$ conidia.cm$^{-2}$ sprays) usually resulted in an antagonistic effect. When both pathogens were simultaneously applied at different combined dosages, usually an additive effect was seen. Interestingly, a number of dead larvae showing signs of co-infections (partially with soft integument and partially mummified) were recorded. However, mixes with lower dosages of both pathogens did not cause significantly higher insect mortalities compared to low dosages of the fungus applied alone. The advantages and disadvantages of the simultaneous applications of NPV and *M. rileyi* aiming at the management of either *A. gemmatalis* or *S. frugiperda* were discussed.

1. Introduction

Noctuids are among the most threatening plant pests worldwide, and their cosmopolitan nature and voracious feeding behavior frequently lead to rapid colonization and substantial losses in many crops (Cunningham et al., 1999; Grzywacz, 2016). Some species, such as *Spodoptera frugiperda* and *Anticarsia gemmatalis*, are particularly harmful to maize and soybean crops, respectively (Gassmann and Clifton, 2016; Sosa-Gómez, 2016). Their adaptability to diverse crop environments and their cosmopolitan nature and voracious feeding behavior frequently lead to rapid colonization and substantial losses in many crops (Abot et al., 1996; Storer et al., 2010; Tabashnik et al., 2012; Binning et al., 2014; Farias et al., 2014). Added to that is the withdrawal of some chemical pesticides from the market and the increasing costs for the development of new and safer chemical products (Chandler et al., 2011; Glare et al., 2012), which has reduced caterpillar control options for growers.

Microbial-based pesticides have long been considered in integrated pest management (IPM) strategies. The nucleopolyhedroviruses (NPVs), genera currently named *Alphabaculovirus* and belonging to the family Baculoviridae (Herniou et al., 2012), and the fungus *Metarhizium rileyi* (Hypocreales: Clavicipitaceae) are well known as “caterpillar killers” and able to naturally control populations of these insects in the field (Ignoffo, 1981; Vimala Devi and Prasad, 2000; Moscardi et al., 2011). Moreover, they are able to cause secondary cycles of infection in caterpillar populations, which is an effective means of long-term pest control (Chandler et al., 2011). Finally, there is no risk (baculoviruses) or little risk (*M. rileyi*) for non-target organisms, and environmental safety justify their reputation as strong candidates in IPM programs. However, both pathogens have limitations when used in stand-alone strategies. NPV can be highly host specific, and in vivo mass production in cannibalistic insects (such as *S. frugiperda*) is not always an easy and
cheap process (Cory and Myers, 2003; Valicente et al., 2013; Grzywacz and Moore, 2016). On the other hand, epizootics caused by M. rileyi frequently appear too late in the season to prevent substantial plant damage by the caterpillars and are highly dependent on host densities (Ignoff et al., 1976; Zabidi et al., 1983). Hence, the development of biopesticides based on more than a single microorganism acting in synergy arises as an alternative to improve efficacy control at more competitive prices.

In the present study, we conducted laboratory experiments with the larval stages of A. gemmatalis and S. frugiperda to investigate whether the simultaneous application of a NPV and M. rileyi strains could result in an advantageous combination and to describe the colonization dynamics of mixed infections. This approach could then be further investigated with the aim of developing combination products for the management of these noctuid pests.

2. Materials and methods

2.1. Inoculum preparation

Strains CG381 and CG1153 of M. rileyi were originally isolated from infected S. frugiperda and S. latifascia larvae, respectively. Both strains are deposited in the Invertebrate-Associated Fungal Collection, at Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil. Strains CG381 and CG1153 were previously shown to be virulent toward S. frugiperda and A. gemmatalis larvae, respectively. Two wild-type baculovirus isolates were used in this study: AgMNPV-79 (=BRM 4997) was isolated from A. gemmatalis larvae by the late Dr. F. Moscardi (Embrapa Soybean) and SfMNPV-6nd (=BRM 5024) from S. frugiperda larvae by Dr. F.H. Valicente (Embrapa Corn and Sorghum), and they are deposited in the Invertebrate Virus Collection, also at Embrapa Genetic Resources and Biotechnology.

Conidia were inoculated on Sabouraud maltose agar with yeast (SMAY) and incubated for 12–14 days at 25 ± 0.5 °C and 12-h photophase. Fresh conidia were scraped with a spatula and suspended in water. Stock suspensions were serially diluted, the concentration adjusted using a Neubauer chamber under a microscope (400×) and immediately used in the bioassays. Conidial viabilities were above 92% for both strains by the time they were used. The virus isolates were multiplied in larvae reared under laboratory conditions (26 ± 0.5 °C, 70% RH and 13-h photophase) on artiﬁcial diet (Greene et al., 1976; Parra, 1996). A. gemmatalis larvae were infected with AgMNPV and S. frugiperda infected with SfMNPV to produce stocks of both viruses for further experiments. The cadavers were kept in the freezer until puriﬁcation of occlusion bodies (OBs). Insect cadavers were homogenized with buffer (1% ascorbic acid; 2% SDS; 0.01 M Tris, pH 7.8; 0.001 M EDTA, pH 8.0), ﬁltered through three layers of cheesecloth and centrifuged at 7000g for 10 min. The pellet was suspended in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0) and again centrifuged at 7000g for 10 min (Maruniak, 1986). The ﬁnal pellet was suspended in distilled water and kept at −20 °C until needed. The OB concentration in the suspension was determined using a Neubauer chamber under a microscope (1000×).

2.2. Interaction between NPV and Metarhizium rileyi in Anticarsia gemmatalis and Spodoptera frugiperda in sequential inoculations

Bioassays were performed with third-instar larvae reared in the laboratory on artiﬁcial diet. Treatments consisted of groups of larvae (S. frugiperda or A. gemmatalis) inoculated with its respective virus and fungal strains alone, with both microbes being used either in a sequential inoculation or in a simultaneous inoculation approach. For fungal inoculations, each group of 12 insects were independently inoculated with 2-mL aliquots of a conidial suspension (5 × 106 conidia mL−1) using a spray tower (Burkard Manufacturing Co Ltd., Rickmansworth, UK) at a pressure of 15 psi (103 kPa), delivering ca. 5 × 103 conidia cm−2. Treated larvae remained for 15 min in contact with the suspension before being individually placed in 24-well tissue culture plates. For inoculation of the virus, leaf discs (1 cm in diameter) were treated with a 10-µL aliquot of suspensions (2.5 × 106 OBs mL−1, equivalent to 2.5 × 104 OBs per leaf disc) and offered to each individual (previously sprayed with 0.05% Tween 80) in the tissue culture plates after complete drop evaporation. Treated leaf discs were the sole source of food in the first 24 h, and larvae were then fed untreated maize (S. frugiperda) or soybean (A. gemmatalis) leaves until the end of the experiment. For the sequential inoculation, larvae were sprayed with the fungus two days after the virus inoculation or virus were offered to fungus-treated larvae two days after spray. In the so-called simultaneous inoculations, groups of third-instar larvae were first sprayed with fungal suspensions and immediately after they were placed in tissue culture plates containing leaf discs with the virus. In each bioassay, a control batch of larvae sprayed with 0.05% Tween 80 was included as a control treatment. The lids of tissue culture plates were lined with moist paper, and plates were kept inside plastic boxes under controlled conditions (26 ± 1 °C and complete darkness) during the entire experimental period. Mortality was assessed daily for ten days. Each treatment was replicated 4 times, totaling 48 insects per treatment. All experiments were performed twice on different dates and with independent insect and microorganism batches.

2.3. Concentration-response of NPV and Metarhizium rileyi in Anticarsia gemmatalis and Spodoptera frugiperda following simultaneous inoculations

Groups of third-instar larvae (S. frugiperda or A. gemmatalis) were inoculated simultaneously with different concentrations of OBs and conidia, up to 4-fold higher than concentrations adopted in the previous assay. The virus was applied to the leaf disc at a maximum concentration of 1 × 107 OBs mL−1 and fractions of 50, 25, and 12.5% of the maximum (full) concentration (leaf discs with 1 × 106 occlusion bodies). The fungus was sprayed on insects at a maximum concentration of 2 × 105 viable conidia cm−2 and fractions of 50, 25, and 12.5% of the full concentration (ca. 2 × 104 conidia cm−2). Both microbes were applied alone at the full concentration as positive controls, whereas the negative control groups were sprayed with 0.05% Tween 80. The number of insects per treatment and the inoculation and evaluation procedures were the same as described in the previous subsection. All experiments were performed twice on different dates and with independent insect and microorganism batches.

2.4. Interaction between NPV and Metarhizium rileyi applied at low dosages against Anticarsia gemmatalis and Spodoptera frugiperda

For the assessment of the virus-fungus interaction in simultaneous inoculation, the pathogens were simultaneously applied at low concentrations, equivalent to 12.5% of the full concentration indicated in Section 2.3. The concentrations of M. rileyi and NPV were 2.5 × 106 conidia mL−1 and 1.25 × 106 OB mL−1, respectively, equivalent to ca. 2.5 × 104 conidia cm−2 or leaf discs with 1.25 × 104 OBs. Experiments were performed twice for both insect species on different dates and with independent insect and microorganism batches, following the same methodology previously described in Section 2.3.

2.5. Colonization dynamics of Anticarsia gemmatalis and Spodoptera frugiperda by NPV and Metarhizium rileyi in simultaneous infections

Dead insects from both sequential and simultaneous infection experiments showing symptoms of fungal infection (mummified bodies) were individually transferred to moistened chambers and kept at 26 ± 0.5 °C and ca. 98% RH. After four days, macro- and microscopic examinations were performed to confirm the infection by M. rileyi. Microscopic examinations of smears from all insects with symptoms of viral infection (soft and/or liquefied bodies) were performed to confirm
the presence of OBs. Cadavers were classified macroscopically into the following categories: mummiﬁed cadaver showing fungal conidiation, cadaver with soft integument and presence of OBs, and cadaver with mixed symptoms (viral and fungal symptoms in different portions of the specimen). Information on quantity of each pathogen in the cadavers in co-infections, although important for considering interactions between pathogens, was not determined.

2.6. Statistical analyses

All experiments were arranged in a completely randomized design and analyses were performed using R Statistical Software (R Development Core Team, 2012). Survival analyses (package ‘survival’) were used to estimate the time to reach 50% insect mortality (ST50). Daily mortality of larvae exposed to the treatments was then analyzed by Cox proportional hazard regression for comparison of survival curves (p < 0.05). Percentages of conﬁrmed mortality by both pathogens were submitted to one-way ANOVA using a logistic regression model using a binomial distribution (Warton & Hui, 2011). Treatments were considered as ﬁxed effect in the model and experiments in time as random effect (block), which were removed from the model when represented a minor component of total variance. GLM selection was previously performed to choose the best model to ﬁt proportional data using the package ‘hnp’ (Moral et al, 2017). In cases where the observed variability was higher or lower than the assumed variance by theoretical distribution (overdispersion), a heterogeneity factor was applied (quasi-likelihood model). Mean values were statistically separated by Tukey’s HSD test at P < 0.05 (function glht in package ‘multcomp”).

Synergistic or antagonistic effects between each NPV and M. rileyi were determined using a procedure adopted by Koppenhöfer and Kaya (1997). The expected proportional additive effect for each NPV-fungus combination on insect mortality (EM) was estimated at six days after inoculation, based on the average ST50 values in the combinations, usually close to six days. EM was calculated by the formula EM = MNPV + MF (1 − MNPV), where MNPV and MF are the observed proportional mortalities caused by each virus and the fungus alone, respectively. Calculated values from a chi-square test (χ2 [MC-EM]/EM, where MC is the observed mortality for each virus-fungus combinations) were compared to the chi-square table value for 1 degree of freedom (χ2 tab = 3.841). Calculated chi-square values (χ2 obs) smaller than the χ2 tab indicate an additive interaction. When χ2 obs exceeded the table value, a possible synergistic (MC-EM = positive value) or antagonistic (MC-EM = negative value) effect between the virus and the fungus was expected at α = 0.05.

3. Results

3.1. Interaction between NPV and Metarhizium rileyi in Anticarsia gemmatalis and Spodoptera frugiperda in sequential inoculations

Differences were observed between survival curves for A. gemmatalis exposed or not to AgMNPV, M. rileyi and the different combinations of the two pathogens (W = 80.97; df = 5; P < 5.551e−16) (Fig. 1A). The control treatment was diﬀerent from all other treatments, and total larval mortality in the former was 8.3%. For each insect, survival curves of the virus applied alone, simultaneously with the fungus or two days before fungal inoculation diﬀered from the treatments where insects were inoculated with M. rileyi alone or two days before the virus. ST50 values varied from 5.95 to 6.77 days (Table 1), and larval mortality was higher than 96.6% for all the treatments with entomopathogens nine days after inoculation. Signiﬁcant diﬀerences were detected among survival curves for S. frugiperda (W = 85.94; df = 5; P = 0) (Fig. 1B). Insects exposed simultaneously to SiMNPV and M. rileyi (Mix 100%) showed shorter ST50 (6.01 days) than with the virus applied alone (7.19 days) or with sequential inoculations of virus and fungus (6.51 and 6.77 days), as shown in Table 1. In the treatment Mix 100%, the percentage of larval mortality was as high as 95.3% ten days after inoculation, while mortality of the virus-treated larvae was only 62.6%. Mortality of control insects was 4.2% for S. frugiperda at the end of the experimental period.

In bioassays with A. gemmatalis, additive interactions between AgMNPV and M. rileyi were observed when the pathogens were applied simultaneously (χ2 obs = 3.02, df = 1) or when the virus was applied before the fungus (χ2 obs = 0.36, df = 1). An antagonistic effect was seen when insects were inoculated with the fungus two days before the virus (χ2 obs = 7.35, df = 1; D = − 25.11%). In bioassays with S. frugiperda, additive interactions were also seen between SiMNPV and M. rileyi applied simultaneously (χ2 obs = 1.29, df = 1). However, the application of the virus two days before (χ2 obs = 19.14, df = 1; D = − 41.31%) or after (χ2 obs = 7.88, df = 1; D = − 26.50%) the fungus had an antagonistic effect.

3.2. Concentration-response of NPV and Metarhizium rileyi in Anticarsia gemmatalis and Spodoptera frugiperda following simultaneous inoculations

Differences were also observed among survival curves for A. gemmatalis exposed or not to simultaneous inoculations of both pathogens (W = 123.6; df = 6; P = 0) (Fig. 1C). The control treatment diﬀered from all the other treatments and insect mortality was 15.6%. The decrease in OBs and conidia concentrations to less than 50% of the full concentration in simultaneous inoculations had a signiﬁcant eﬀect on survival of third-instar A. gemmatalis larvae. ST50 values of mixtures with either 25 or 12.5% of the full concentration were similar to the pathogens applied alone but longer than for mixtures with either 50% or 100% of the full concentration (Table 2). Differences were also observed among survival curves of NPV-fungus combinations for S. frugiperda (W = 78.09; df = 6; P < 8.882e−15) (Fig. 1D). Lower ST50 values were detected for all the NPV-fungus combinations (< 6.51 days) when compared to the virus applied alone (7.39 days). Mortality of insects exposed to the combination of the two pathogens at 50% of the maximum concentration (Mix 50%) did not diﬀer from that caused by the full concentration (Mix 100%), and ST50 was signiﬁcantly shorter than with either pathogen applied alone (Table 2). Mortality of control insects was only 2.1% for S. frugiperda after nine days. An additive interaction was detected for A. gemmatalis (χ2 obs = 0.17, df = 1) in the simultaneous use of NPV and the fungus. The combined use of SiMNPV and M. rileyi showed a synergistic eﬀect for S. frugiperda (χ2 obs = 5.51, df = 1; D = 18.07%).

3.3. Interaction between NPV and Metarhizium rileyi applied at low dosages against Anticarsia gemmatalis and Spodoptera frugiperda

Survival curves for A. gemmatalis exposed or not to simultaneous inoculations of both pathogens at low dosages were diﬀerent (W = 24.7; df = 3; P = 1.766e−05) (Fig. 1E). Differences in survival curves were also seen for S. frugiperda (W = 17.9; df = 3; P = 0.00047) (Fig. 1F). In both cases, lower ST50 values were detected for the virus applied alone when compared to the NPV-fungus combinations and the fungus applied alone at low dosages (Table 3). In both bioassays, no larval mortality was recorded in the control treatments.

An additive interaction between AgMNPV and M. rileyi was clear when the pathogens were applied simultaneously at low dosage (χ2 obs = 1.50, df = 1) in bioassays with A. gemmatalis. (χ2 obs = 0.36, df = 1). On the other hand, in bioassays with S. frugiperda the combination of SiMNPV and M. rileyi had an antagonistic eﬀect (χ2 obs = 10.01, df = 1; D = − 23.30%).

3.4. Colonization dynamics of Anticarsia gemmatalis and Spodoptera frugiperda by NPV and Metarhizium rileyi in simultaneous infections

The number of dead larvae colonized by the virus and/or fungus differed between treatments in sequential inoculations (P = 405.82;
When tested alone, AgMNPV was considerably as effective in colonizing *A. gemmatalis* larvae as *M. rileyi*. Increased fungal infection in *A. gemmatalis* exposed to both pathogens was observed only when the fungus was applied two days before the virus, although the mortality achieved with the latter was not lower. In this particular case, the percentage of dead larvae mummified by the fungus was 28.8%, whereas the proportion of cadavers with soft integument due to the virus was as high as 37.5%. Mummification occurred in < 10% of the cadavers following simultaneous application or when the virus was applied two days ahead (Fig. 2A). When the virus and the fungus were simultaneously applied at different concentrations, differences in confirmed mortality were also observed between treatments ($F = 891.33; \ df = 5, 42; P = 2.988 \times 10^{-12}$). At all combinations, cadavers with soft bodies were more frequent than dead larvae mummified by *M. rileyi* (Fig. 2C).

For *S. frugiperda*, differences on the confirmed mortality were also observed between treatments when the pathogens were applied in sequential inoculations ($F = 419.56; \ df = 4, 35; P = 8.148 \times 10^{-5}$). The number of cadavers mummified by *M. rileyi* was almost 7-fold higher than soft-integument larvae when the fungus was applied first, although mortality rates were similar when the two pathogens were applied together, or the viral infection was higher when the virus was applied two days in advance (Fig. 2B). With simultaneous inoculation, confirmed mortality in the different treatments was similar ($F = 268.73; \ df = 5, 42; P = 0.1289$), and mortality caused by the fungus (mummified cadavers) never surpassed the rates seen for the virus (soft integument) used in combination, as previously observed in the experiment with *A. gemmatalis* (Fig. 2D).

Differences on the confirmed mortality were also observed among treatments when the pathogens were applied at low dosages for both *A.*
of both insect species with symptoms of viral infection (soft or liquefied tissues). The two pathogens in different insect species can significantly differ in their interaction, as reported by Cox proportional hazard regression (p < 0.05). ND – not determined (insect mortality did not reach 9% in the control treatments).

### Table 2
Mean survival time (ST50) of *Anticarsia gemmatalis* and *Spodoptera frugiperda* third-instar larvae exposed to simultaneous application of different dosages of NPV and *Metarhizium rileyi* strains.

<table>
<thead>
<tr>
<th>Host insect</th>
<th>Treatments</th>
<th>ST50 (days)</th>
<th>Confidence interval (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. gemmatalis</em></td>
<td>Control</td>
<td>ND&lt;sup&gt;1&lt;/sup&gt;</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>AgMNPV</td>
<td>6.04</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>CG1153</td>
<td>6.47</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>AgMNPV + CG1153</td>
<td>5.95</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>Mix 100%</td>
<td>5.98</td>
<td>c</td>
</tr>
<tr>
<td><em>S. frugiperda</em></td>
<td>Control</td>
<td>ND</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>SMNPV</td>
<td>7.19</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>CG381</td>
<td>6.20</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>SMNPV + CG381</td>
<td>6.77</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>CG381 + SMNPV</td>
<td>6.51</td>
<td>cd</td>
</tr>
<tr>
<td></td>
<td>Mix 100%</td>
<td>6.01</td>
<td>e</td>
</tr>
</tbody>
</table>

<sup>1</sup> Entomopathogens were applied alone (NPV or CG), or the fungus was sprayed 2 days after (NPV + CG) or before (CG + NPV) the virus; except for treatment Mix 100%, in which the two pathogens were simultaneously applied using full dosages (5 × 10⁶ conidia mL⁻¹ and 1.5 × 10⁶ OB mL⁻¹).

<sup>2</sup> Time-response values followed by the same letter within host species are not significantly different by Cox proportional hazard regression (p < 0.05).

<sup>3</sup> ND – not determined (insect mortality did not reach 9% in the control treatments).

### Table 3
Mean survival time (ST50) of *Anticarsia gemmatalis* and *Spodoptera frugiperda* third-instar larvae exposed to simultaneous application of low dosages of NPV and *Metarhizium rileyi* strains.

<table>
<thead>
<tr>
<th>Host insect</th>
<th>Treatments</th>
<th>ST50 (days)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Confidence interval (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. gemmatalis</em></td>
<td>Control</td>
<td>ND&lt;sup&gt;1&lt;/sup&gt;</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>AgMNPV Low</td>
<td>8.07</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>CG1153 Low</td>
<td>7.09</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>Mix Low</td>
<td>7.12</td>
<td>c</td>
</tr>
<tr>
<td><em>S. frugiperda</em></td>
<td>Control</td>
<td>ND</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>SMNPV Low</td>
<td>7.44</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>CG381 Low</td>
<td>6.94</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>Mix Low</td>
<td>7.00</td>
<td>c</td>
</tr>
</tbody>
</table>

<sup>1</sup> Entomopathogens were applied alone (NPV or CG) or simultaneously using low concentrations (2.5 × 10⁶ conidia mL⁻¹ and 1.25 × 10⁶ OB mL⁻¹).

<sup>2</sup> Time-response values followed by the same letter within host species are not significantly different by Cox proportional hazard regression (p < 0.05).

### 4. Discussion

We described the interaction between NPV and *M. rileyi* strains when used together (sequential or following simultaneous applications) against two important lepidopteran pests, *A. gemmatalis* and *S. frugiperda*. In general, NPV-fungus combinations had either additive or antagonistic effects on mortality, whereas the prevalence of a given pathogen in co-infections depended on the host species and inoculation strategy. When *M. rileyi* was applied two days earlier, mortality of *S. frugiperda* caused by the fungus was considerably higher than those caused by the virus, but in simultaneous applications, the virus was more effective in colonizing the larvae than its fungal counterpart. The prevalence of the virus in NPV-fungus combinations was more evident for *A. gemmatalis*.

Interestingly, this is the first report of a dual infection by a NPV and *M. rileyi*, in both *S. frugiperda* and *A. gemmatalis* larvae. Although competition between the pathogens for the host tissues seems likely, in some cases both pathogens were able to colonize the same insect body. The combined use of NPVs and other entomopathogens has been considered as a potential strategy against lepidopteran pests, and according to Lacey et al. (2015), a better understanding of how NPVs interact with other biological control agents can be one of the keys for the development of new products or strategies, enhancing their overall performance.

Differently from entomopathogenic bacteria, which are capable of killing their hosts in a few hours, the slower killing speed of NPVs and *M. rileyi* strains allowed concomitant infections in our experiments, likely favored by their distinct modes of action but with similar times for infection. Co-infections by more than one pathogen species naturally occur among insects, leading to an independent coexistence in the host or resulting in negative (antagonism) or positive (synergism) interactions (Cox, 2001; Read and Taylor, 2001). The combined use of NPV and *Bacillus thuringiensis* against lepidopteran pests has been reported, usually resulting in additive effects on insect mortality (Kalantari et al., 2014; Maghолli et al., 2013; Qayyum et al., 2015). Interactions between viruses and entomopathogenic fungi have also been reported in the past (Ferron and Hurpin, 1974; Lecuona and Alves, 1988; Malakar et al., 1999), but limited to just a few hosts and pathogen.
species. For instance, co-infections were already reported in *Lymantria dispar* larvae simultaneously treated with a NPV and *Entomophaga maimaiga* (Malakar et al., 1999), and in *Melolontha melolontha* larvae exposed to an entomopoxvirus and a species of the genus *Beauveria* (Ferron and Hurpin, 1974). In the latter, the combined use of entomopathogens increased the susceptibility of virus-infected larvae to the fungus. In our study, simultaneous use of NPV and *M. rileyi* did not show any synergistic effect on mortality of either *S. frugiperda* or *A. gemmatalis* larvae. Instead, an additive effect was commonly seen in combinations of viruses and fungal strains, and many individuals that were not infected by the virus eventually died due to fungal infections.

Our results suggest that the combined use of these pathogens in a single bioproduct does not provide benefits for the management of some lepidopteran pests if control efficacy were the only criterion to be considered. Simultaneous infestation of more than one lepidopteran species is not uncommon in various crops and NPVs have a very narrow host range, not rarely limited to a single insect species (Ignoffo and Garcia, 1985; Szewczyk et al., 2009; Grzywacz, 2016). Despite the relatively narrow host specificity of *M. rileyi* (Boucias et al., 1982; Ignoffo and Boucias, 1992; Kulkarni and Lingappa, 2002), selected strains that infect multiple lepidopteran species could potentially kill insects that escape from viral infection. Finally, co-infected larvae from simultaneous inoculation of NPV and *M. rileyi* could potentially spread both polyhedra and conidia in the environment, increasing the inocula and promoting secondary outbreaks in the field. Alternatively, these pathogens could show antagonism under field conditions, which would decrease potential secondary cycling compared to the pathogens alone application. The enzootic occurrence of both pathogens in crops could also be important for the management of insect populations resistant to chemical insecticides or Bt-crops.

Fig. 2. Confirmed mortality (% ± SE) of *Anticarsia gemmatalis* (A, C and E) and *Spodoptera frugiperda* (B, D and F) larvae following either different combinations or simultaneous applications of their respective NPV and *Metarhizium rileyi* strains. Columns followed by the same letter are not significantly different (P < 0.05).
Infection of fungus-inoculated *S. frugiperda* followed by NPV inoculation greatly affected the number of larvae killed by the latter. The fungus also contributed with 24–43% of the confirmed mortality when larvae were simultaneously inoculated with the two pathogens. In this case, the highly virulent strain CG381 was able to systemically colonize the host before complete occupation of the insect tissues by the virus. In addition, the mutant strain SfMNPV-6nd used in our study is reported to take a longer period of time to kill *S. frugiperda* larvae when compared to other highly virulent viral strains (Vieira et al., 2012), increasing the chances for fungal colonization. Interestingly, we observed the presence of OBs of SfMNPV in internal tissues of mummified cadavers in some of the double infected larvae, indicating that the virus was able to successfully colonize some host cells even in the presence of the fungus. Conversely, there was no evidence of replication of *M. rileyi* in cadavers or tissues with symptom of viral infection. As a matter of fact, co-infections in lepidopteran larvae simultaneously inoculated with up to two different fungi and a granulovirus has been reported (Pauli et al., 2018).

The contribution provided by *M. rileyi* in the NPV-fungus mixture shown in our study with *S. frugiperda* was not clearly detected in the simultaneous infection of *A. gemmatalis*. Survival times in simultaneous inoculations not always matched with the time to death of virus-treated larvae.

**Fig. 3.** *Spodoptera frugiperda* (A and B) and *Anticarsia gemmatalis* (C) larvae with mixed symptoms after co-infection by their respective NPV and *Metarhizium rileyi* strain; microscopic examination of internal body tissues from the borderline regions of co-infected *Spodoptera frugiperda* showing both viral (intracellular) and fungal structures (D); *Anticarsia gemmatalis* cadavers showing symptom of fungal (E) and virus (F) infection in dual infected larvae.
larvae but the virus always caused higher confirmed mortality, regardless of the concentration of either pathogen. In other words, when AgMNPV and M. rileyi invade the host at the same time, this virus, known for its high virulence (Moscardi & Sosa-Gómez, 2007), becomes the major cause of larval mortality in all tested dosages. The same trend was previously observed in mixed infections by granulosis viruses and *Beauveria bassiana* on *Diatraea saccharalis* (Lecuona and Alves, 1988; Pauli et al., 2018), in which the viruses prevailed, and inversely by NPV and *Entomophasa maimaga* on *Lymnantria dispar* larvae, in which the fungus was observed in the majority of cadavers (Malakar et al., 1999).

This seems to be related to the ability of one of the pathogens to rapidly infect and colonize the host at the dosages tested. Nevertheless, a tendency of increasing larval mortality by the fungus was detected when dosages of both pathogens in the mixture was reduced. For instance, the tendency of increasing larval mortality by the fungus was detected when the virus infected and colonized the host at the dosages tested. Nevertheless, a ten-fold lower NPV infection, with 82.7% in the treatment Mix 25%, but 60% in Mix 12.5%.

In our study, perhaps the lowest dosages of the virus induced the infection, with 82.7% in the treatment Mix 25%, but 60% in Mix 12.5%.

Gómez, 2000), ca. 1.7–2.5 times lower than the higher concentration used in the laboratory assay. In fact, it has been shown that field applications of this virus effectively reduce *A. gemmatalis* populations below damaging levels, but negatively influence the magnitude of natural M. rileyi epizootics (Moscardi et al., 1981; Zabidi et al., 1983). On the other hand, the field rate used for SfMNPV aiming at *S. frugiperda* control is estimated to be 1.3–2 × 10^6 OBs mL^-1 (1.5 × 10^10 OBs ha^-1) (Valicente et al., 2013), very similar to what was used in our laboratory study (only 1.2–1.9 times lower).

Antagonism is not unexpected when two distinct pathogens compete for nutrients or host sites, as seen in our study, especially when NPV was applied two days before the fungus, since the latter only caused minimal mortality levels under this circumstance. Insects colonized by the fungus or showing mixed symptoms appeared later and their number increased for the lowest dosages tested. It is likely that the longer time for a systemic insect colonization by the AgMNPV at a lower inoculum level allowed the occupation of some tissues by the fungus. Viral infection in some tissues can be delayed, as reported in *Trichoplusia ni*, since the infected midgut was able to recover from the NPV invasion by sloughing off diseased cells and replacing them with new healthy cells (Keddie et al., 1989; Engelhard and Volkman, 1995). In our study, perhaps the lowest dosages of the virus induced the sloughing off of infected midgut cells, giving the fungus a chance.

Regarding co-infections, portions of an insect cadaver occupied by one of the pathogens were not suitable for the other, generating a clear division of the host infected tissues. Notably, there was a morphological intersection, shown by light microscopy, in which both pathogens could be seen. In bioassays with *A. gemmatalis* and *S. frugiperda*, synergism was not seen when NPVs and *M. rileyi* strains were combined, and at lower dosages the control seen was not improved compared to low dosages of the fungus applied alone. In conclusion, our results highlight the importance of similar studies with other hosts to better understanding the interactions between distinct microorganisms in order to achieve synergism and all the benefits of co-infections.

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