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CABI Agriculture and Bioscience (2022) 3:24
https://doi.org/10.1186/s43170-022-00094-7

CABI Agriculture and Bioscience

RESEARCH

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Fungal and viral entomopathogens as a combined strategy for the biological control of fall armyworm larvae in maize

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Abstract

Background: The fall armyworm *Spodoptera frugiperda* is one of the major pests in maize crops, causing important production losses. The pest has rapidly spread worldwide, generating an urgent need to develop efficient and sustainable strategies for its control. In this work, the potential of integrating nucleopolyhedrovirus- (NPV) and the fungus *Metarhizium rileyi* to control *S. frugiperda* larvae was evaluated under laboratory, greenhouse, and field conditions.

Methods: The mortality of *S. frugiperda* larvae was evaluated after the application of NPV and *M. rileyi* alone or in combination using three concentrations (high, medium and low) under laboratory conditions. Then, two greenhouse trials using maize plants were carried out to evaluate the effect of individual or combined applications of NPV and *M. rileyi* on *S. frugiperda* mortality (first trial) and fresh damage (second trial). Finally, a trial under field conditions was conducted to evaluate the performance of the treatment selected in the greenhouse assay.

Results: The combined use of NPV: *M. rileyi* applied simultaneously showed an additive effect in laboratory, causing higher larval mortality than the biocontrol agents used separately. This effect was evident in the mixtures using the concentration levels high:medium, medium:medium, and medium:high. Under greenhouse conditions, the use of a 50:50 ratio of the two entomopathogens also caused higher larval mortality and a significantly reduced insect damage to plants. Finally, under field conditions, the individual or sequential application of NPV and *M. rileyi* using 100% of their recommended doses, and the simultaneous application of both entomopathogens at 50% of their recommended doses, significantly reduced the recent foliar damage to levels under the threshold for economic losses (30% fresh damage) while the damage reached 43% when control measures were not used.

Conclusion: The combined application of NPV and *M. rileyi* (two biocontrol agents with different mode of action) demonstrated an additive effect that allows to reduce to half their recommended application doses. In this context, the integration of both entomopathogens is a promising strategy to manage *S. frugiperda,* contributing to improve the economic feasibility of biological control tools for the sustainable fall armyworm management.

Keywords: Fall armyworm, Nucleopolyhedrovirus, *Metarhizium rileyi*, Combined use, Maize crop, Biological control

Background

Spodoptera frugiperda (Lepidoptera: Noctuidae) or fall armyworm (FAW) is a moth species native to tropical and subtropical regions of the Americas, which was described first in the United States, causing damage in western Florida (Nagoshi et al. 2012). The FAW is a polyphagous

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pest with 353 host plants belonging to 76 families, with preference for the *Poaceae* including maize (*Zea mays*), forage grasses (*Panicum* spp.), sugar cane (*Saccharum officinarum*), rice (*Oryza sativa*) and sorghum (*Shorgum* sp.) (Casmuz et al. 2010; Montezano et al. 2018). This polyphagous condition along with its high reproductive capacity, feeding voracity and the ability to migrate long distances (a moth can fly 100 km per night) (Song et al. 2020) facilitates the wide distribution of the pest in different geographic regions. In addition to that, climatic variability has an effect over insect populations changing the development rate, the duration of the life cycle and ultimately, the insect populations survival (Du Plessis et al. 2020).

Recently, *S. frugiperda* has also become more relevant due to its report in the African continent in 2016, (Goergen et al. 2016) and its rapid spread to more than 40 countries. In mid-2018 the pest was reported in India (Kalleshwaraswamy et al. 2018) and in 2019 in China (Jing et al. 2019; Sun et al. 2019), spreading rapidly to other Asian countries. By May 2020, the insect was reported at 11 regions of Queensland, three regions of the northern territories and three regions of Western Australia, as well as at Timor-Leste and Mauritania (Wan et al. 2021).

The use of chemical insecticides is one of the main strategies for controlling FAW in the world; however, several agrochemicals have high toxicity and interfere with the persistence and effectiveness of natural control agents. In addition, *S. frugiperda* has developed resistance to several commercially available insecticides, including some *Bacillus thuringiensis* toxins (Bolzan et al. 2019; Lira et al. 2020).

The important economic losses caused by *S. frugiperda* in several regions, generate the need for efficient control tools, able to be integrated into crop management programs. Among the natural enemies of *S. frugiperda*, pathogens as fungi and viruses represent an alternative to chemical insecticides. For example, several isolates of *Metarhizium rileyi* (Ascomycota: Clavicipitaceae) (Kepler et al. 2014) (formerly *Nomuraea rileyi*) obtained from naturally infected larvae of *S. frugiperda* collected in maize crops in Colombia, were screened for their insecticidal activity against the pest (Bosa et al. 2004). The most virulent strain (Nm006) was later formulated as an emulsifiable concentrate, biopesticide that demonstrated high potential to control FAW larvae in maize (Grijalba et al. 2018). Among the entomopathogenic viruses, those belonging to the baculovirus family (Baculoviridae) are the most used to develop viral biopesticides and the *S. frugiperda* multiple nucleopolyhedrovirus (SfMNPV) has been widely studied for the control of FAW in different crops in America (Barrera et al. 2011; Haase et al. 2015; Behle et al. 2012). Specifically in Colombia, one isolate of this nucleopolyhedrovirus codified as SfMNPV003 was developed as a biopesticide (wettable powder) containing microencapsulated viral particles (Villamizar et al. 2010; Gómez et al. 2013). This biopesticide demonstrated high efficacy against the FAW in maize crops under laboratory and field conditions (Gómez et al. 2013; Barrera et al. 2017).

Despite the potential of both Biological Control Agents (BCAs) for the control of S. *frugiperda*, there are some biological (speed of action, host range) or economic (high production costs by in vivo production systems) limitations that need be overcome (Grzywacz and Moore 2017) to ensure the adoption by farmers. In this sense, the combined use of entomopathogenic agents with different mode of action could have additive or synergistic effects that can result in an enhanced insecticidal activity associated with the reduction in the minimum effective doses. This effect was recently reported by Lobo et al. (2019), who assessed the interaction between NPV and *M. rileyi* against *Anticarsia gemmatalis* and *S. frugiperda* under laboratory conditions. An additive effect was seen when both pathogens were simultaneously applied using different doses, and the authors concluded that further studies are needed to better understand the interactions between the microorganisms and to achieve all the benefits of co-infections.

In this context, the present work aimed to study the effect of applying two BCAs isolated from *S. frugiperda* (NPV and *M. rileyi*), on their insecticidal activity against the pest under laboratory, greenhouse, and field conditions.

Materials and methods

Insects

Larvae of *S. frugiperda* were obtained from a laboratory colony established from eggs collected in a maize crop (Tolima—Colombia). Each larva was reared individually on a piece of wheat germ-based semi-synthetic diet contained in a plastic container (15 mL) (Gómez et al. 2010). The colony was maintained under controlled conditions at 26 ± 2 °C, 60% relative humidity and 12:12 h (light:dark) photoperiod.

Production of NPV and *M. rileyi*

The viral isolate SfMNPV003 [RGE0263 (No. 200) and RGE0163 (No. 141) contract for access to genetic resources and their derived products in Colombia] and the fungus *Metarhizium rileyi* Nm006 [RGE0229-2 (No. 168) contract for access to genetic resources and their derived products in Colombia] are preserved in the Germplasm Bank of Microorganisms with Interest for

Biological Control of AGROSAVIA, located at "Tibaitatá" Research Center (Mosquera, Colombia).

Production of SfMNPV003 was carried out by orally inoculating third instar *S. frugiperda* larvae obtained from a laboratory colony, with a viral suspension containing 10^6 occlusion bodies per milliliter (OBs/mL) (Ruiz et al. 2015). Virus-killed insects were homogenized in 0.1% sodium dodecyl sulphate (SDS) solution (w/v) and the homogenate was filtered through a fine a sterilized cheesecloth to remove insect debris. The resulting OB suspension was quantified using an improved Neubauer hemocytometer (Hawksley, Lancing, UK) under light microscopy at ×400 (Santos et al. 2014).

Metarhizium rileyi isolate Nm006 was mass produced following the methodology described by Grijalba et al. (2018). Metallic trays with 150 g of rice and 150 mL of protein hydrolysate (8%) were inoculated with 20 mL of conidia suspension (1×10^6 conidia/mL). After 10 days of incubation at 25 °C, the fungus sporulated and dry conidia were harvested by sweeping them with a brush from the substrate surface.

Effect of mixtures of NPV and *M. rileyi* on *S. frugiperda* mortality under laboratory conditions

To evaluate the insecticidal activity of the BCAs alone or in simultaneous application, a biological test using three concentrations of each pathogen was carried out: high (1×10^6 OBs or con/mL), medium (1×10^5 OBs or con/mL) and low (1×10^4 OBs or con/mL). The treatments are described in Table 1.

Bioassays were performed with second-instar *S. frugiperda* larvae from a colony reared under laboratory conditions on artificial diet. Larvae were inoculated with each pathogen alone or combined. *Metarhizium rileyi* was topically inoculated by placing a 1 µL drop of the fungal suspension on the dorsal side of each larva. SfMNPV003 was inoculated by the droplet feeding method using a drop of 1 µL of the viral suspension. For the simultaneous inoculations, larvae were fed with the virus first and immediately after, they were exposed to the fungus. Each inoculated larva was transferred to individual 15 mL plastic cups containing a fragment of a castor oil plant (*Ricinus communis*) leaf, previously disinfected with 0.5% hypochlorite and used as a natural diet to feed the larvae (Grijalba et al. 2018). Groups of 15 cups were placed in plastic boxes (experimental units) and incubated under controlled conditions (25 ± 2 °C; RH: 60% ± 5). Larval mortality was recorded after 5 days, and signs of disease related with the possible cause of death (virus or fungi) were recorded.

The experiment was conducted with a randomized complete block design with three replicates per treatment, each one corresponding to a plastic box with 15 s instar larvae. Larvae without any treatment were used as control treatment. Mortality data were corrected for control mortality and reported as efficacy according with the Schneider-Orelli formula (Zar 1999):

$$Efficacy(\%) = \frac{(A - C)}{(100 - C)} \times 100$$

where A = Mortality in the treatment and C = Mortality in the control.

For combination treatment, the nature of the pathogen interaction (synergistic, additive, or antagonistic effects) was also determined using the procedure described by Koppenhöfer and Kaya (1997). For that, the expected additive proportional mortality M_E for the virus-fungus combinations was calculated by using the following equation:

$$M_E = M_V + M_F(1 - M_V)$$

where M_V and M_F are the individual mortalities caused by virus or fungus, respectively. Then, the combined effect was estimated by:

$$x^2 = \frac{(M_{VF} - M_E)^2}{M_E}$$

where M_{VF} is the observed mortality of the combination virus + fungus. The chi-square values were then compared with the table of chi-square probabilities for 1 DF. If the calculated x^2 value was lower than the value in the table, an additive effect between the two agents was considered. But if the obtained result exceeded the critical chi-square value, a nonadditive effect

Table 1 Treatments used for evaluation of BCAs applied alone or simultaneously

Treatment	NPV	*M. rileyi*
T1	High	Low
T2	Low	High
T3	Medium	High
T4	High	Medium
T5	Medium	Low
T6	Low	Medium
T7	Medium	Medium
T8	Low	Low
T9	High	–
T10	Medium	–
T11	Low	–
T12	–	High
T13	–	Medium
T14	–	Low
T15	Control (without application)	

(synergistic or antagonistic) was suggested. Subsequently, if $D = M_{BN} - M_E$, had a positive value, a significant interaction could be considered synergistic, and if D had a negative value, a significant interaction could be antagonistic. The effect between the virus and the fungus was determined at $P < 0.05$.

Effect of different mixtures of NPV and *M. rileyi* on *S. frugiperda* mortality under greenhouse conditions

The insecticidal activity of mixtures of SfMNPV003 and *M. rileyi* (Nm006) mixtures against second-instar larvae of *S. frugiperda* was evaluated on maize plants under greenhouse conditions at Mosquera (Cundinamarca, Colombia. 18 ± 5 °C; RH: 70% ± 10). The experiment was conducted with a randomized complete block design with four treatments and three replicates per treatment. Each experimental unit consisted of ten 470 mL pots with one maize plant, giving a total of 30 plants per treatment. Treatments corresponded to three mixture of both BCAs in different proportions that were calculated based on the dose previously recommended for the field application of each entomopathogen (100%), corresponding to 8×10^{11} OBs/ha for SfMNPV003 (Barrera et al. 2017) and 1.3×10^{12} con/ha for *M. rileyi* Nm006 (Grijalba et al. 2018) (Table 2).

Twenty days after emergence of plants (V2 phenological stage), a manual sprayer was used to apply the treatments (2 mL/plant). The volume applied per plant was calculated based on a planting density of 75,000 maize plants per hectare (Martínez Uribe et al. 2017) and an application volume of 150 L per hectare (Moscardi and Sosa-Gómez 2007).

One hour later, each plant was infested with three second instar larvae of *S. frugiperda*, which were transferred with a soft brush. A total of 30 larvae per replicate and 90 larvae per treatment were used. Two days later, all larvae were collected and transferred to individual 15 mL cups containing a fragment of disinfected *R. communis* leaf as feeding substrate (Grijalba et al. 2018). Plastic cups were maintained under laboratory conditions (Temperature: 25 ± 2 °C; Relative Humidity: 60% RH) and the number of alive and dead larvae were recorded at 5 days. To document the presence of fungal sporulation, disease signs were monitored daily until day 9 after application.

Table 2 Different ratios for BCAs mixtures

Treatment	NPV	*M. rileyi*
T1	75% (2.0×10^{11} OBs/ha)	25% (3.2×10^{11} con/ha)
T2	50% (4.0×10^{11} OBs/ha)	50% (6.5×10^{11} con/ha)
T3	25% (6.0×10^{11} OBs/ha)	75% (9.7×10^{11} con/ha)
T4	Control (without application)	

Mortality data were corrected for control mortality and reported as efficacy according with the Schneider-Orelli formula (Zar 1999).

Effect of individual, simultaneous and sequential application of NPV or *M. rileyi* on recent damage caused by *S. frugiperda* on maize plants under greenhouse conditions

The effect of applying both BCAs using different strategies on the damage caused by *S. frugiperda* on maize plants was evaluated using the mixing ratio selected in the previous assay (NPV 50%: *M. rileyi* 50%).

The experiment was conducted under greenhouse conditions at Mosquera (Cundinamarca, Colombia. 18 ± 5 °C; RH: 70% ± 10), with a randomized complete block design with six treatments and three replicates per treatment. Each experimental unit consisted of ten 470 mL pots with one maize plant giving a total of 30 plants per treatment. Treatments are described in Table 3.

Twenty days after plants emerged (V2 phenological stage), the first application of treatments was carried out by using the same methodology described previously. One hour later, each applied plant was infested with three larvae of *S. frugiperda* in second instar, for a total of 30 larvae per replicate and 90 larvae per treatment. The larvae were gently transferred onto the surface of the leaf by using a soft brush. The recent damage in the plants caused by *S. frugiperda* was evaluated 2, 4, 6 and 8 days after application (DAA). Recent damage was defined as the presence of areas with scraping, holes and fresh frass on the newest leaf and was measured in a binary scale (presence or absence of fresh damage) (Lasa et al. 2007; Gómez et al. 2013; Toepfer et al. 2021). After recording the plant damage on day 8, the second application was carried out following the methodology previously

Table 3 Description of treatments for evaluation of individual, combined and alternate application of BCAs

Treatment	First application		Second application	
	BCA	Dose	BCA	Dose
T1	NPV	8.0×10^{11} OBs/ha	NPV	8.0×10^{11} OBs/ha
T2	*M. rileyi*	1.3×10^{12} con/ha	*M. rileyi*	1.3×10^{12} con/ha
T3	NPV	8.0×10^{11} OBs/ha	*M. rileyi*	1.3×10^{12} con/ha
T4	*M. rileyi*	1.3×10^{12} con/ha	NPV	8.0×10^{11} OBs/ha
T5[a]	NPV	4.0×10^{11} OBs/ha	NPV	4.0×10^{11} OBs/ha
	M. rileyi	6.5×10^{11} con/ha	*M. rileyi*	6.5×10^{11} con/ha
T6	Control (without application)			

[a] Suspension containing a mixture of both BCAs using 50% of the field recommended doses

described and the recent damage was assessed in days 10, 12 and 14 after the first application.

Effect of individual, simultaneous and sequential application of NPV or *M. rileyi* on recent damage caused by *S. frugiperda* on maize plants under field conditions

The field trial was carried out in a maize crop located at "La Europa" Farm in the municipality of Espinal (Tolima, Colombia. 25 ± 3 °C; RH: 75% ± 5). (N 03° 48′14″ W 73° 18′58″). Mechanized corn planting was conducted using the hybrid 'FNC 8134'. Experimental plots were arranged in a randomized complete block design, with four replicate plots per treatment. Each plot was 2 m long, comprising four rows with eight plants per row. Plots were separated from each other by three buffer rows of untreated plants. Plots at the edges of the experimental area were surrounded by additional rows of untreated plants to reduce edge effects. The crop was managed with the conventional agronomic practices of irrigation, fertilization and weed control for maize.

The experiment involved four treatments (Table 4), including the treatment selected in the greenhouse assay (T1).

The BCAs suspended in water were applied with a 20 L backpack sprayer (Royal Condor Ref. No. 1898157), spraying the surface of the leaves and specially the whorl leaves of young plants. Treatments were applied in a volume of 150 L per hectare.

Foliar damage under natural infestation by fall armyworms was evaluated weekly until 35 days after the emergence of the plants (V4 phenological stage), by sampling all plants in the four rows. The recent damage caused by *S. frugiperda* was defined as the presence of areas with scraping, holes and fresh frass on the newest leaf and was measured with a binary scale (presence or absence of fresh damage) (Lasa et al. 2007; Gómez et al. 2013; Toepfer et al. 2021) and expressed as a percentage. The recent damage data was used to calculate the percentage of efficacy for each treatment by using the Henderson-Tilton formula, which compares the number of plants with damage in the treatments and the absolute control:

$$Efficacy(\%) = \left(1 - \left(\frac{Co_1 * Tr_2}{Co_2 * Tr_1}\right)\right) * 100$$

where Co_1 corresponds to the number of plants with recent damage in the control before application, Tr_2 is the number of plants with recent damage in the treatment after application, Co_2 is the number of plants with recent damage in the control after application and Tr_1 is the number of plants with recent damage in the treatment before application (Henderson and Tilton 1955).

Table 4 Scheme for application of BCAs under field conditions

Treatment	First application (day 7)		Second application (day 21)	
	BCA	Dose	BCA	Dose
T1[a]	NPV	4.0×10^{11} OBs/ha	NPV	4.0×10^{11} OBs/ha
	M. rileyi	6.5×10^{11} OBs/ha	*M. rileyi*	6.5×10^{11} OBs/ha
T2	NPV	8.0×10^{11} OBs/ha	NPV	8.0×10^{11} OBs/ha
T3	*M. rileyi*	1.3×10^{12} con/ha	*M. rileyi*	1.3×10^{12} con/ha
T4	Control (without application)			

[a] Suspension containing a mixture of both BCAs

Statistical analysis

Results were subjected to a Bartlett test ($P < 0.05$) to determine homoscedasticity and Shapiro Wilks test ($P < 0.05$) to assess data normality. Results were then subjected to repeated-measures analysis of variance (ANOVA) and Least Significant Difference (LSD) test ($P < 0.05$) or Tukey's test ($P < 0.05$) using the statistical software Statistix version 8.1 (Analytical Software, Tallahassee, FL, USA).

Results

Effect of mixtures of NPV and *M. rileyi* on *S. frugiperda* mortality under laboratory conditions

Fungal infection was characterized by presence of mycelium or pale green sporulation, and rigid body, while viral infection was characterized by soft body, pink discoloration or rupture of the epidermis. In some cases, a combined infection was observed with the characteristics of viral infection, but with the appearance of mycelium at the apical or caudal end.

There was no larval mortality in the control after five days. Larval mortality caused by the SfMNPV003 applied at three different concentrations was 26% (low concentration), 43% (medium concentration) and 63% (high concentration), and statistical differences were detected among the three treatments ($F = 15.2$; DF = 2,6; $P = 0.0045$). The fungus *M. rileyi* Nm006 caused 10% (low concentration), 13% (medium concentration) and 47% (high concentration) mortality, being the result obtained with the high concentration, significantly higher than those obtained with the low and medium concentrations ($F = 22.2$; DF = 2,6; $P = 0.0017$).

When the highest concentration of NPV or *M. rileyi* were combined with the lowest concentration of the other BCA, infection symptoms exhibited by dead larvae corresponded exclusively to those produced by the pathogen in higher concentration in the mixture and mortality percentages did not differ from those obtained with these pathogens applied alone (Fig. 1A and B). However, mixing the medium concentration of each pathogen

with the high or the medium concentration of the other BCA, significantly increased the mortality in comparison with the pathogens applied individually (Fig. 1C, D, and G). The mixtures that used the lowest concentration of a pathogen with the medium or the lowest concentration of the other BCA did not increase the mortality of *S. frugiperda* larvae in comparison with the pathogens applied alone (Fig. 1E, F and H).

The chi-square test for virus + fungus combinations showed significantly higher efficacy than the pathogens alone in the treatments: high:medium (− 940.95), medium:high (− 2060.78) and medium:medium (− 638.8). Since the calculated chi-square value was lower than the one in the table for 1 DF (3.8415), it could be said that the effect shown by the combination of both BCAs was additive.

All larvae treated with BCAs showed retarded growth compared with healthy larvae in the control treatment (Fig. 2A). Signs of infection in dead larvae were among treatments inoculated with fungus, virus, or mixtures. Larvae killed by the action of the fungus showed a progressive stiffness until complete mummification with cadavers completely covered with white mycelia (Fig. 2B), that later sporulated with powdery green conidia. The virus infection caused changes in the color of the integument with an increase in glossiness. The larvae showed a swollen body with flaccid appearance and were extremely fragile (Fig. 2C). Larvae that died due to mixed infection showed swollen bodies with typical viral infection signs, accompanied with fungus hyphae emerging from the cadaver (Fig. 2D).

Effect of different mixing ratios of NPV and *M. rileyi* on *S. frugiperda* mortality under greenhouse conditions

At day five post application, the efficacy of treatments varied between 13 and 63% (Table 5), being T3 significantly lower ($F = 8.18$; DF = 2,8; $P = 0.0193$) than T1 and T2, which corresponded to higher virus concentrations.

Regarding the signs of infection, most larvae infected with the mixtures exhibited those typical of a viral disease. However, in some cases the combined infection was evident, with larvae simultaneously exhibiting signs of infection caused by the two pathogens, as previously observed (Fig. 2D). The signs of infection in the cadavers at day 9 post inoculation were recorded and expressed as percentage (Fig. 3). Viral infection signs were found to be predominant in T1 and T2, with few larvae showing fungal development (< 14%). Treatment 3 corresponding to the mixture containing the highest level of fungus (75%) showed a higher proportion of larvae with fungal (33.3%) and mixed (8.6%) signs in comparison with T1 and T2 (Fig. 3).

Under greenhouse conditions, T2 that corresponds to NPV 50%: *M. rileyi* 50% showed the higher insecticidal efficacy and was selected to evaluate its effect using individual, simultaneous and sequential application of BCAs.

Effect of individual, simultaneous and sequential application of NPV or *M. rileyi* on recent damage caused by *S. frugiperda* on maize plants under greenhouse conditions

The control treatment presented 100% of plants with recent damage caused by *S. frugiperda* from day 2nd to day 10th after the first application (Fig. 4). Then, this value decreased to 96.6% and 83.3% at 12 and 14 days respectively. All treatments where BCAs were applied presented more than 90% of the plants with recent damage at 2, 4 and 6 DAA. At day 8 before carrying out the second application, the plants from treatments T1, T3 and T5 where the virus was used in the first application presented the lowest recent damage levels (16.7–26.7%), which were significantly different ($F = 66.8$; DF = 5,12; $P < 0.0001$) in comparison with the control and treatments T2 and T4 where only fungus was applied (93.3% recent damage).

At day 10, two days after the second application of BCAs, all treatments presented less than 50% of plants with recent damage and were significantly different from the control ($F = 99.4$; DF = 5,12; $P < 0.0001$). No damage (0%) was detected in treatment T1 where only virus was used in both applications, and this level remained unchanged until the experiment finished. The same tendency was maintained at days 12 and 14 after the first application (corresponding to 4 and 6 days after the second application), with all treatments exhibiting significantly lower recent damage (< 50%) than the control. Treatment T2 and T4 presented the same recent damage level at both evaluation dates with 43.3% and 23.3% at 12 and 14 DAA. Two weeks after the first application (14 DAA), the recent damage was less than 5% in all treatments where virus was used for the first application (T1, T3 and T5), values that were significantly lower ($F = 140$;

(See figure on next page.)

Fig. 1 Efficacy of combined application of NPV and *M. rileyi* against *S. frugiperda* larvae under laboratory conditions at five days after treatment. Data are mean ± SE (n = 3). Treatments with the same letter did not present significant differences according to LSD ($P < 0.05$). **A** NPV High: *M. rileyi* Low ($F = 34$; DF = 2,8; $P = 0.0005$). **B** NPV Low: *M. rileyi* High ($F = 6$; DF = 2,8; $P = 0.037$). **C** NPV Medium: *M. rileyi* High ($F = 5.17$; DF = 2,8; $P = 0.0396$) **D** NPV High: *M. rileyi* Medium ($F = 58.5$; DF = 2,8; $P = 0.0001$). **E** NPV Medium: *M. rileyi* Low ($F = 31$; DF = 2,8; $P = 0.0007$) **F** NPV Low: *M. rileyi* Medium ($F = 4.33$; DF = 2,8; $P = 0.0685$) **G** NPV Medium: *M. rileyi* Medium ($F = 44.3$; DF = 2,8; $P = 0.0003$). **H** NPV Low: *M. rileyi* Low ($F = 10.5$; DF = 2,8; $P = 0.011$)

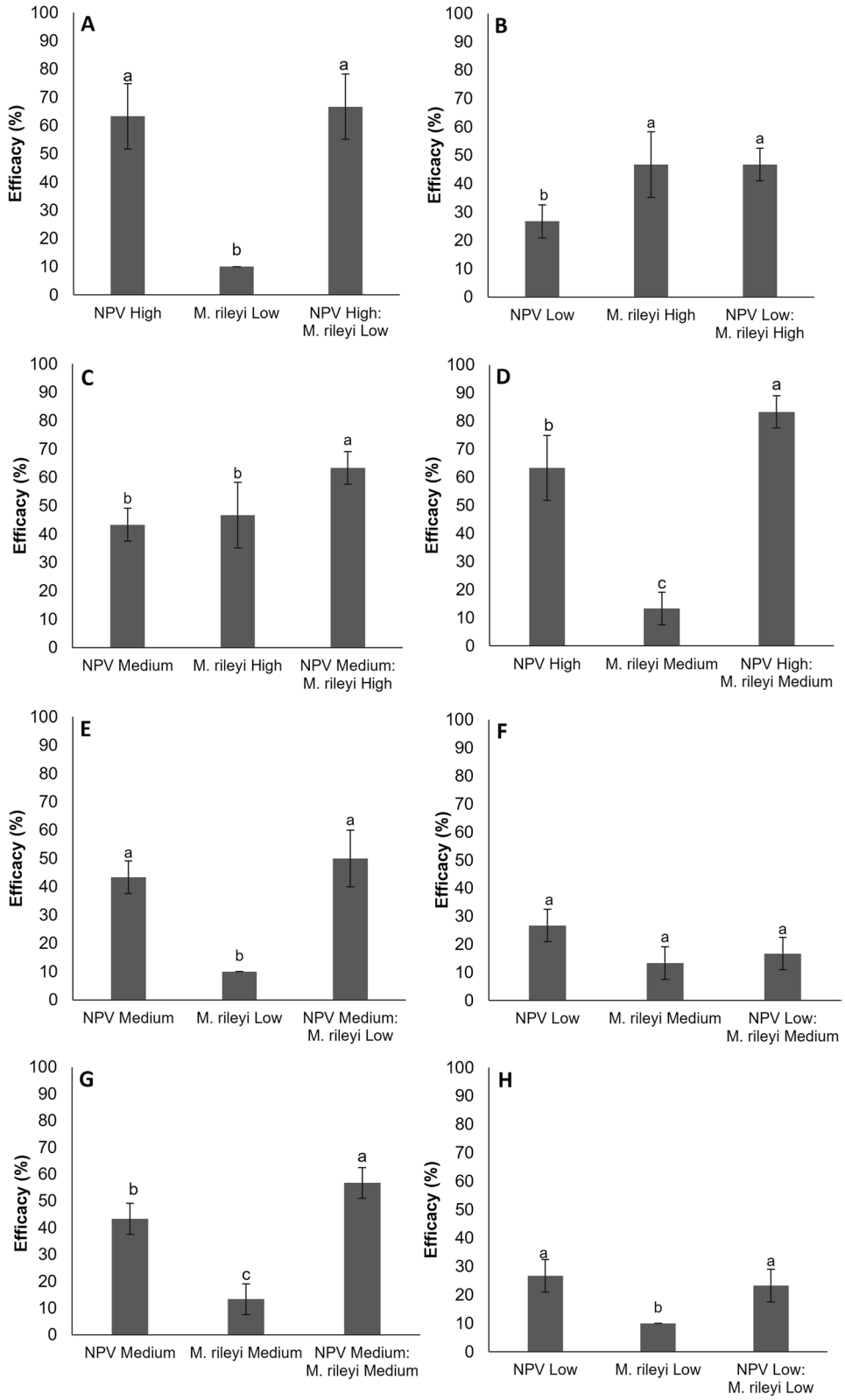

Fig. 1 (See legend on previous page.)

Fig. 2 Signs of infection in treated larvae **A** Healthy larvae from control treatment. **B** Fungus infected larvae. **C** Virus infected larvae. **D** Mixed infection by fungus and virus

Table 5 Efficacy of the mixtures of NPV and *M. rileyi* against *S. frugiperda* under greenhouse conditions 5 days after application

Treatment	NPV (%)	*M. rileyi* (%)	Efficacy (%)*
T1	75	25	52.4 a
T2	50	50	63.3 a
T3	25	75	13.2 b

*Treatments with the same letter did not present significant differences according to LSD ($P < 0.05$)

DF = 5,12; $P < 0.0001$) than those obtained with treatments T2 and T4 where fungus was used first (Fig. 4).

Effect of individual, simultaneous and sequential application of NPV or *M. rileyi* on recent damage caused by *S. frugiperda* on maize plants under field conditions

The initial recent damage ranged from 0 to 6.2% (Fig. 5). At days 21 and 28 post-emergence of the plants, the level of damage caused by *S. frugiperda* was higher than 30%, the established economic injury level in maize crops (Fernández 2002).

The recent damage level increased to 13.5% in all treatments on day 14 and reached its maximum level at 21 days post-emergence of the plants, with values between 35 and 55% (Fig. 5) and without statistical differences between treatments (Day 14: $F = 1.62$; DF = 6,13; $P = 0.2202$. Day 21: $F = 0.47$; DF = 6,13; $P = 0.7598$. Day 28 $F = 0.54$; DF = 6,13; $P = 0.7110$).

The highest level of recent damage was detected in the absolute control treatment, where no measure for *S. frugiperda* control was used (Fig. 5). It is important to note that besides the high level of recent damage, a high number of plants with damage in the whorl was also observed in these plots.

On day 35 post-emergence of the plants, the recent damage ranged between 17.7 and 24.4% in all the treatments where the application of BCAs was carried out, values that were significantly lower than that obtained in the absolute control that reached 48.8% ($F = 11.5$; DF = 6,19; $P = 0.0009$) (Fig. 5).

The efficacy of each treatment was calculated using the recent damage data. Values ranged from 84 to 91%, with no significant differences detected among them ($F = 0.16$; DF = 6,17; $P = 0.9732$), suggesting that all treatments similarly controlled the pest.

Discussion

Integrated Pest Management (IPM) is a sustainable approach that can help to minimize the damage of *S. frugiperda* on crops, by combining biological, cultural, and chemical tools. In this sense, BCAs can be an option to implement as part of an effective IPM strategy against fall

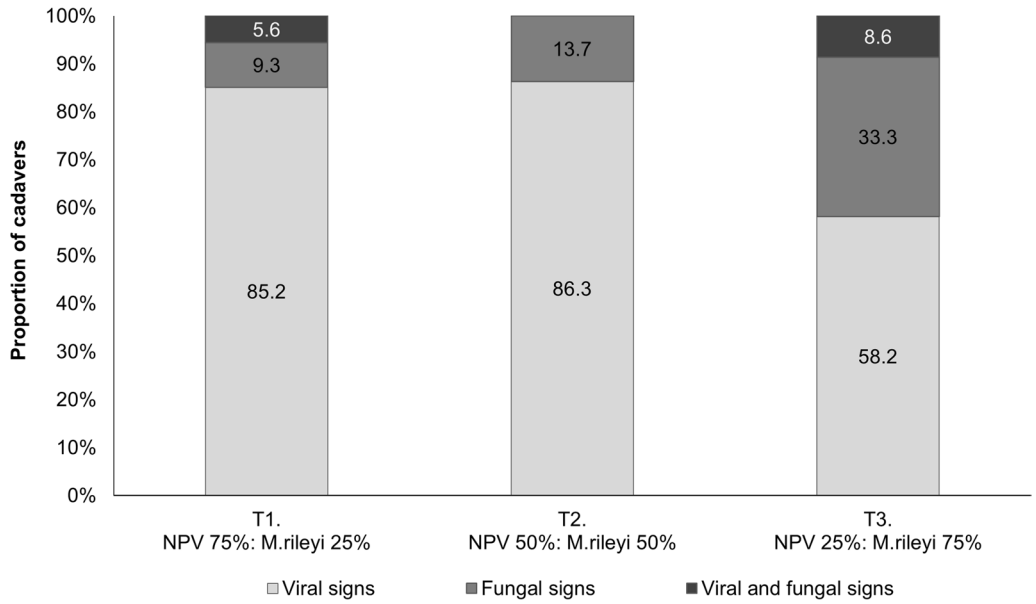

Fig. 3 Percentage of *S. frugiperda* cadavers with signs of viral, fungal, or mixed infection at day 9 post infection. Data are mean (n = 3)

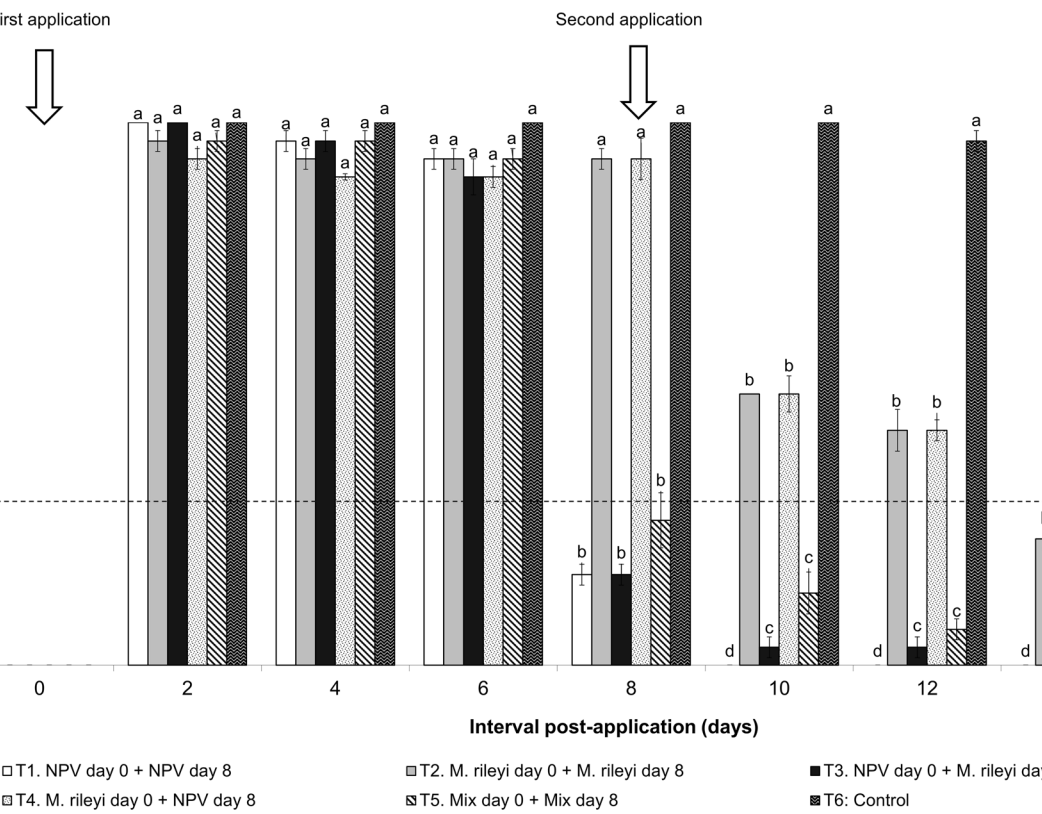

Fig. 4 Reduction of recent damage caused by *S. frugiperda* larvae in maize plants after the application of BCAs NPV (SfMNPV003) and *M. rileyi* (Nm006) under greenhouse conditions. Data are mean ± SE (n = 3). The statistical analysis was performed separately for each evaluation time. Treatments with the same letter did not present significant differences according to Tukey ($P < 0.05$). Error bars indicate standard errors of the mean. The dotted line indicates the economic injury level for *S. frugiperda* in maize (Fernández 2002)

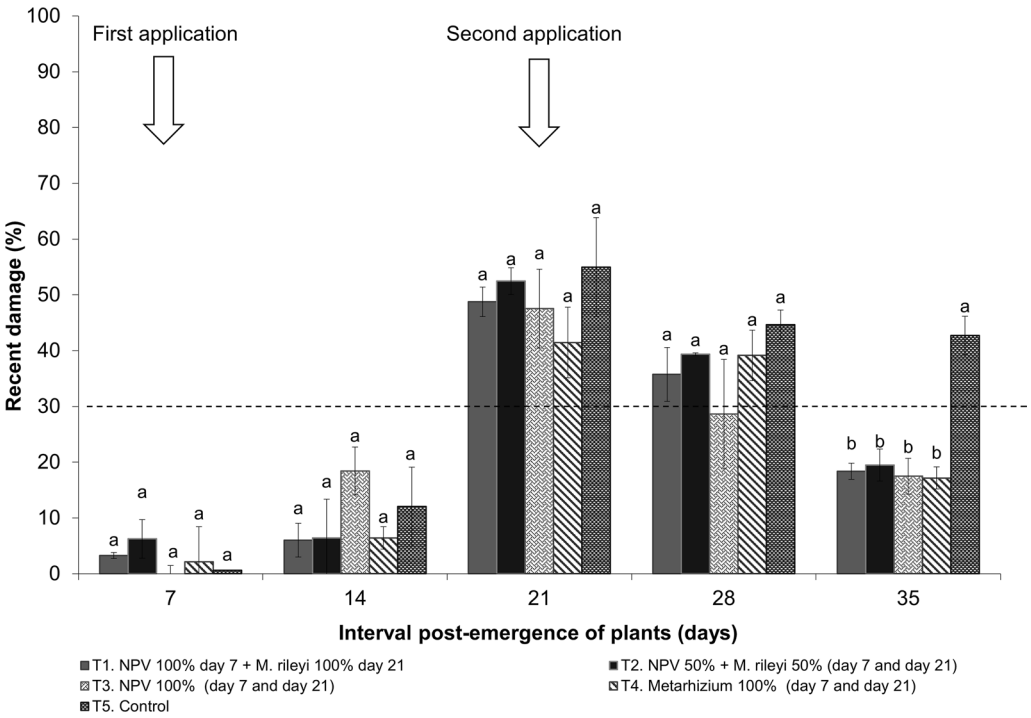

Fig. 5 Reduction of recent damage caused by *S. frugiperda* larvae in maize plants after the application of BCAs NPV (SfMNPV003) and *M. rileyi* (Nm006) under field conditions. Data are mean ± SE (n = 3). The statistical analysis was performed separately for each evaluation time. Treatments with the same letter did not present significant differences according to Tukey ($P < 0.05$). Error bars indicate standard errors of the mean. The dotted line indicates the economic injury level for *S. frugiperda* in maize (Fernández 2002)

armyworm (Wan et al. 2021). A great diversity of natural enemies including entomopathogens associated with this pest and with different mechanisms to reduce the population of *S. frugiperda* larvae has been reported (Guo et al. 2020). Their combined use could result in better control of the pest considering that different BCAs with different modes of action against the same pest may enhance the results by independently targeting different points of vulnerability in the insect host (Narciso et al. 2019; Gulzar et al. 2021). This strategy has been explored against different insect pests by using the combination of bacteria and fungi (Narciso et al. 2019; Magholi Fard et al. 2020) and viruses and fungi (Lobo et al. 2019) showing potential to improve the efficacy at a reduced cost.

It is important to consider several factors to achieve the maximum efficacy when combining two or more biocontrol agents, such as (synergistic or additive) or negative (antagonist) effects that may occur due to the interaction between them, as well as the appropriate mixture ratio and timing of application to enhance effectiveness (Narciso et al. 2019). For example, Lobo et al. (2019), demonstrated that when *M. rileyi* was applied two days before NPV, fungal infection prevailed. But when applied simultaneously, the virus was more effective in colonizing than the fungus. The same authors reported an additive effect between NPV and *M. rileyi* when applied simultaneously on *S. frugiperda*. However, when the virus was applied two days before or after the fungus, the effect was antagonistic. It should be noted that these experiments were carried out under laboratory conditions being necessary to validate these effects using maize plants under field conditions.

In the present work, the laboratory results indicated that the mixture of SfMNPV003 and *M. rileyi* Nm006 with higher insecticidal potential corresponded to the simultaneous application of half of the concentration recommended for each entomopathogen (Mixture NPV medium: *M. rileyi* medium, Fig. 1G). This mixture showed an additive effect suggesting that both BCAs applied together might act independently of each other and neither of them benefits or harms the other (Koppenhöfer and Kaya 1997). Mixtures with lower or higher concentrations of the pathogens were not suitable for use, because they did not enhance the efficacy or because they performed the same as using the high concentration of BCAs, showing no advantage. This result agrees with that reported by Pauli et al. (2018), who indicated a lack of synergism when mixing at low doses

granulovirus, *Beauveria bassiana* and *M. anisopliae* to control *Diatraea saccharalis* (Lepidoptera: Crambiidae) larvae. There are few observations where synergistic effects are evident in nucleopolyhedroviruses (Tanada 1985; Morris et al. 1996) or fungi and its combination with viruses (Pauli et al. 2018).

The application of mixtures of NPV and *M. rileyi* under greenhouse conditions demonstrated that mixing them in a 50:50 proportion can increase the mortality of *S. frugiperda* larvae in the initial days after the application (day five post application) (Table 4), confirming the additive effect of both BCAs. Although the virus was more effective in colonizing the larvae cadavers compared to *M. rileyi*, the fungus also contributed with 9.3–33% of the confirmed mortality when larvae were simultaneously inoculated with the two BCAs. The killing speed of NPVs and *M. rileyi* strains allow concomitant infections, likely favored by their distinct modes of action but with similar times for infection (Lobo et al. 2019). It is important to note that SfMNPV003 is slightly faster to kill *S. frugiperda* larvae with a mean lethal time of 5.9 days (Barrera et al. 2013), while the lethal time of *M rileyi* is 6.3 days (Bosa et al. 2004).

The treatment NPV and *M. rileyi* mixed in 50:50 proportion of their recommended doses was then selected to evaluate its effect on the recent damage reduction in maize plants under greenhouse conditions. Individual, simultaneous, and alternate applications of both BCAs under greenhouse conditions significantly reduced the recent damage caused by *S. frugiperda* in maize plants in comparison with non-treated plants that maintained > 80% damage. However, the most efficient treatments were those in which SfMNPV003 was used for the first application, alone at its recommended dose (100%) or in a mixture with *M. rileyi* at half of its recommended dose (50%). These treatments reduced and maintained the recent damage below the established economic damage threshold of 30% (Fernández 2002) from 8 days after application until the end of the experiment (14 days after application) (Fig. 4). These results could suggest that the main cause of larval control during the week after the first application was the viral infection, which is possibly related to its faster mode of action. The use of 100% SfMNPV003 for both applications under greenhouse conditions, completely controlled the pest with no recent damage after the second application.

Under field conditions, the recent damage in the control treatment reached 55% exceeding the economic injury level (30%), which could result in 13% to 30% reduction in yield (Ayala et al. 2013; Jeger et al. 2017). The combined use of both BCAs (simultaneously or sequential) efficiently controlled the pest (> 85% efficacy), performing similarly to each BCA used alone. Similar results were reported by Biaggioni et al. (2020) who evaluated the efficacy of an oil dispersion (OD) preparation that combines NPV and *M. rileyi* against lepidopteran soybean pests. In this work, the authors found that the action of the mixture (NPV + *M. rileyi*) induced satisfactory levels of overall larval mortality, although its performance was similar to the formulation containing each pathogen alone.

The present study demonstrates for the first time the potential of using mixtures of NPV and *M. rileyi* to control *S. frugiperda* in maize plants at the seedling stage, a combination that allows reducing 50% of the dose previously recommended for each BCA (Barrera et al. 2017; Grijalba et al. 2018). For SfMNPV003 the dose could be reduced from 8×10^{11} to 4×10^{11} OBs/ha and for *M. rileyi* Nm006 from 1.3×10^{12} to 6.5×10^{11} con/ha. Another possibility is to sequentially use both entomopathogens during the crop cycle, always starting with the virus applied few days after plant emergence, considering that a faster mode of action is needed to reduce the risk of young plants being killed due to voracious *S. frugiperda* defoliation (Sisay et al. 2019). This strategy reduces the quantity of each entomopathogen needed in one crop cycle.

Although the use of each entomopathogen alone at its full dose achieved the same levels of pest control as the combined use (sequential or simultaneous), using the two entomopathogens has many advantages.

From an economic point of view, the feasibility of using biopesticides based on these pathogens increases, since less product would be required to achieve adequate pest control percentages, emphasizing the reduction in NPV volumes considering that the high labor required for its propagation plays a critical role in its availability and cost (Ruiz et al. 2015). Another advantage of using virus and fungi in combination is the potential of managing several pests at the same time with the same products. Both pathogens have been extensively studied against *S. frugiperda* (Sosa-Gómez 2017; Guo et al. 2020) and it is well known that SfMNPV is specific to *S. frugiperda* (Simón et al. 2004), while *M. rileyi* is pathogenic to other lepidopteran pests found affecting maize and soybean crops as *Helicoverpa* sp., *Chloridea virescens* and *Anticarsia gemmatalis* (Lobo et al. 2019; Biaggioni et al. 2020). Additionally, the varying acute efficacy (lethal time) of each entomopathogen would represent an advantage, considering that applying NPV always in the early stages of the crop would allow a faster control of an initial high population of the pest, and the mixture with the fungus would favor a long-term control because both pathogens are capable of persisting in the environment causing secondary cycles of infection (Cruz et al.

1997; Aguirre et al. 2009; Barrera et al. 2017; Espinel-Correal et al. 2019), especially the use of *M. rileyi* that produces important epizootics on *Spodoptera* species for its high dispersion capacity due to the presence of pulverulent sporulation (Montecalvo and Navasero 2021). Finally, and considering that nucleopolyhedroviruses are more resistant to environmental conditions than fungi such as *M. rileyi* (data not shown), combining the use of the two entomopathogens can enhance field performance and compensate for the loss of efficacy or the dependence on the host density-dependent (Fuxa and Richter 1999).

Conclusions

The findings from the current study demonstrated the feasibility of using two pathogens with different modes of action (SfMNPV and *M. rileyi*) to control the insect pest *S. frugiperda* in maize crops. Co-inoculation of both BCAs at half of their recommended doses, or their sequential application, resulted in an additive effect. This effect is highly promising and could be integrated into an improved IPM program for the fall armyworm. However, further studies directed to develop a dual-action biopesticide that combines NPV and *M. rileyi* are needed, as well as more studies to refine the recommendations for its application and integration with other methods of control.

Acknowledgements
We thank to Carlos Molina from Fenalce (Federación Nacional de Cultivadores de Cereales, Leguminosas y Soya, Colombia) for his invaluable support in the logistic and design of the bioassays.
We thank to Buenaventura Monje, Claudia Milena Mesa, Jhon Pablo Vargas, David Triana and Javier Pulido (Corporación Colombiana de Investigación Agropecuaria, Agrosavia) for the support in bioassays development in laboratory, greenhouse, and field.

Author contributions
LVR conceived and designed the project and applied for funding; JGV, PCO, GBC, CEC and LVR performed the laboratory, greenhouse and field assays, and conducted the data analysis; JGV and LVR prepared the manuscript with substantial input from GBC, PCO and CEC, and JGV and LVR reviewed and edited the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by the grant from the Ministerio de Ciencias, Tecnología e Innovación—Minciencias (Colombia) contract 802–2011, and by the Federación Nacional de Cultivadores de Cereales, Leguminosas y Soya—Fenalce (Colombia) that provided the maintenance of the field plots, as well as human talent for the experimental crops. The Colombian Corporation of Agricultural Research (Agrosavia) provided physical and human resources. This work was carried out under the RGE0229-2 (No. 168), RGE0263 (No. 200) and RGE0163 (No. 141) contract for access to genetic resources and their derived products in Colombia.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 30 June 2021 Accepted: 4 April 2022
Published online: 29 April 2022

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