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Potential Impact of Chemical Fungicides on the Efficacy of *Metarhizium rileyi* and the Occurrence of *Pandora gammae* on Caterpillars in Soybean Crops

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Abstract
Entomopathogenic fungi may play a crucial role in the regulation of caterpillar populations in soybean crops, either through natural occurrences or applied as mycopesticides. In the present work, we reported the naturally occurring entomopathogenic fungus *Pandora gammae* attacking the caterpillar *Chrysodeixis includens*, with infection rates in field trials ran in two consecutive years in the 10–35% range. As many chemicals are potentially harmful to entomopathogenic fungi, this work aimed to investigate the potential impact of two chemical fungicides (azoxystrobin + benzovindiflupyr and trifloxistrobina + prothioconazole) used to control soybean rust (*Phakopsora pachyrhizi*) on the natural occurrence of *P. gammae* and *Metarhizium rileyi*, as well as the efficacy of the latter fungus applied as different formulations against the soybean caterpillars *Anticarsia gemmatalis* and *C. includens*. Under laboratory conditions, fungicides used at field-recommended rates had a considerable negative impact on the germinability of *M. riley* on the medium surface, and all tested formulations did not protect conidia from damage by these chemicals. This harmful effect also impacted host infectivity, as the larval mortality owing to this fungus was reduced by 30–40% compared to that of the fungicide-free treatments. In field trials conducted in two subsequent years, unformulated and formulated *M. rileyi* conidia applied to soybean plants produced primary infection sites in caterpillar populations after a single spray. Spraying unformulated or formulated *M. rileyi* conidia following fungicide application on plants did not affect host infection rates over time. Moreover, the use of *M. rileyi*-based formulations or chemical fungicide did not interfere with the natural infection rates by *P. gammae* on its host, *C. includens*. Although a higher degree of exposure to non-selective fungicides can negatively affect fungal entomopathogens, a single foliar application of fungicides may be harmless to both *M. rileyi* and *P. gammae* in soybean fields. Additionally, this work showed that naturally occurring wasps and tachnids also play an important role in the regulation of *A. gemmatalis* and, notably, *C. includens*, with parasitism rates above 40–50% in some cases.

Keywords Entomophthorales · Compatibility · Microbial control · Soybean caterpillars

Introduction

Entomopathogenic fungi play an important role in the natural regulation of different insect pests in several crops, particularly in the application of strategies for conservation biological control or integrated pest management (IPM) [1].

The natural occurrence of the fungus, *Metarhizium rileyi* (Farlow) Kepler, Rehner and Humber (Hypocreales: Clavicipitaceae), on caterpillar populations has been an important component for the management of lepidopteran pests of soybean on the American continent [2, 3]. The velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Erebidae), and the soybean looper, *Chrysodeixis includens* (Walker, 1858) (Lepidoptera: Noctuidae), are among the most harmful pests and are both susceptible to *M. rileyi* infection. Other entomopathogenic fungi can infect caterpillars, such as *Cordyceps tenuipes* and entomophthoralean species [3, 4]. Most reports on entomophthoralean species within the genus *Pandora* refer to natural infections on aphid populations [5, 6], although this group of fungi

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is also known to infect hosts in other taxonomic orders, including *A. gemmatalis* and *C. includes* [7, 8]. Likewise, several parasitoids of these two pests have been reported on soybeans [8, 9]. Together with parasitic wasps and tachinid flies, entomophatogenic fungi usually represent the greatest portion of natural enemies of lepidopteran pests in soybean fields [8–10].

Changes in land use, from monoculture farming to continuous multiple crop systems, and the emergence of several new pests and diseases have led to the overuse of agrochemicals in soybeans [11]. Chemical fungicides have been demonstrated to be particularly harmful to *M. rileyi* [3, 12–14], and their use to control soybean rust caused by the pathogen, *Phakopsora pachyrhizi* (Pucciniales: Phakopsoraceae), has recently increased. The lack of compatibility between many chemical pesticides and fungal-based products poses a risk for the incorporation of biocontrol strategies in conventional agriculture. Therefore, biopesticide companies seek to develop new market-acceptable formulations to integrate them with other pest control practices that are routinely performed by farmers. Simple formulations may protect conidia from abiotic stressors, as previously reported for species in the *Metarhizium anisopliae* complex [15–17], including the negative effects of some chemical fungicides [18]. For the fungus *M. rileyi*, only experimental low-cost formulations have been tested under laboratory conditions [19–21], greenhouse [22], and field conditions [9, 23]. However, information on how these formulations may protect *M. rileyi* conidia from the negative effects of the chemical fungicides used in soybean crops is unavailable.

In the present study, we assessed the efficacy of low-cost formulations of *M. rileyi* under laboratory and field conditions against soybean caterpillars and the potential negative effects of soybean rust fungicides on entomopathogen development. In addition, we evaluated the potential impacts of chemical fungicides and *M. rileyi* applications on the natural occurrence of the entomophthoralean fungus *Pandora gammae* (J. Weiser) Humber on *C. includens* populations in the field.

Material and Methods

Conidia Production and Formulation

The fungal strain used in this study, *M. rileyi* CG1153, was deposited in the Invertebrate-Associated Fungal Collection of EMBRAPA Genetic Resources and Biotechnology, Brasília, DF, Brazil. This strain has high virulence to *A. gemmatalis* and infects *C. includens* larvae [9]. Aerial conidia were produced on cooked parboiled rice loaded into polypropylene bags inoculated with a 4-day-old fermented broth containing this fungus. The liquid culture was produced in Sabouraud maltose yeast medium (SMY 40 g maltose, 10 g peptone, and 20 g yeast extract per liter of distilled water) on an orbital shaker at 26 ± 0.5 °C in the dark. After 10–12 days of incubation (26 ± 1 °C), the fungus-colonized rice was pre-dried at room temperature for 48 h, and conidia were harvested by sieving. The conidia were then dried by exposure to drierite (anhydrous calcium sulfate) for 2 days at 24 ± 1 °C in hermetic glass containers (3 L). Unformulated dry conidia had approximately 1×10^{11} conidia g^{-1} and 6–6.5% water content (equivalent to ca. 0.2 water activity). After 48 h of incubation at 26 ± 0.5 °C in the dark, conidia germination percentage on SMY amended with agar (SMAY) was greater than 92% by the time of use.

Oil dispersion (OD) was prepared by mixing dry conidia with an oil-based preparation composed of vegetal oil and surfactants, with a final concentration of 1×10^9 viable conidia g^{-1}. Water-dispersible granules (WG) were produced by mechanical agglomeration of conidia and inert compounds (corn starch, maltodextrin, and distilled water) into granules with approximately 0.75 mm of diameter and a final concentration of 5×10^9 viable conidia g^{-1}. The OD and WG preparations are described in detail by Lopes and Faria [24]. Wettable powder (WP) was obtained by mixing dry conidia (5% w/w) with corn starch (45% w/w), an aluminosilicate mineral (zeolite clinoptilolite, 45% w/w), and the suspension stabilizer carboxymethyl cellulose (5% w/w), with a final concentration of 5×10^9 conidia g^{-1}. *C. includens* larvae used in all lab bioassays were obtained from an insect colony kept at EMBRAPA Genetic Resources and Biotechnology and reared on artificial diet [25].

Bioassays of the *Metarhizium rileyi* Formulations Against *Chrysodeixis includens* Larvae

OD, WG, and WP formulations and non-formulated conidia (NF) were suspended in distilled sterile water plus Tween 80 (0.05% v/v) and adjusted to a final concentration of 1×10^7 viable conidia mL^{-1}. Groups of 16 third-instar *C. includens* larvae were independently sprayed with 2 mL of conidial suspensions using a spray tower (Burkard Manufacturing Co Ltd., Rickmansworth, UK) delivering approximately 6×10^3 conidia cm^{-2}. After 15 min, the larvae were individually placed in 16-well plastic trays and fed on soybean leaves for 10 days. Larval mortality was assessed daily beginning the third day after spraying. Larvae treated with only distilled water plus Tween 80 (0.05%) were used as a negative control (C). Dead larvae in all treatments were placed in moistened chambers to determine the cause of death. Each treatment was performed in triplicate, and the entire experiment was performed a total of two times using different fungal batches.

In Vitro and In Vivo Effects of Chemical Fungicides on Formulated and Unformulated *Metarhizium rileyi* Conidia

First, the effect of the chemical fungicides, azoxystrobin + benzovindiflupyr (Elatus®WG, Syngenta) and trifloxistrobina + prothioconazole (Fox®SC, Bayer), on conidial germination was evaluated in solid culture medium. The commercial fungicides were diluted in distilled water at the recommended concentrations of 100 g·100 L^{-1} and 400 g·100 L^{-1}. A 50 µL droplet was placed on the SMAY surface in Petri dishes and kept in a laminar flow hood for 15 min until complete droplet evaporation. Approximately 5 µg of *M. rileyi* formulations (OD, WG, and WP) or NF was placed in a glass tube with 10 mL of distilled water and vortexed. Thereafter, a 20 µL aliquot of the suspension was placed onto the dried fungicide droplet on the medium, air dried in a laminar flow hood, and incubated at 26 ± 0.5 °C in the dark. Conidial viability was assessed by direct microscopic observation (400 × magnification) of 100 conidia per sample after 48 h of incubation. Germinated conidia were defined as those with germ tubes longer than the width of an ungerminated conidium. Formulated and unformulated conidia applied to medium without fungicides were used as positive controls. Each treatment was repeated four times, and the entire experiment was repeated on different dates with different fungal batches.

The influence of both chemical fungicides on the infectivity of *M. rileyi* NF and OD formulations was assessed in third-instar *C. includes* larvae. Soybean leaf discs (4-cm diameter) were immersed in a 5 mL solution of each fungicide at the same concentrations as described for the in vitro study and left to dry in a fume hood. Thereafter, leaf discs were treated with a 2 mL conidial suspension (1×10^7 viable conidia mL^{-1}) using a spray tower delivering approximately 6×10^3 conidia cm^{-2}. Groups of 16 larvae were fed treated leaf discs for 24 h and then individually placed in 16-well plastic trays. Larvae were fed untreated soybean leaves for the duration of the experiment, and mortality was assessed daily up to the tenth day post-spray. Leaf discs immersed in distilled water plus Tween 80 (0.05%) or fungicide suspensions that were not treated with the fungus were used as negative controls. Dead larvae in all treatments were placed in moistened chambers to determine the cause of death. Each treatment had four trays, and the entire experiment was repeated on different dates with different fungal batches.

Natural Occurrence of Fungal Entomopathogens in a Soybean Field and Species Identification

An epizootic occurrence of an entomophthoralean fungus on *C. includens* was detected in an experimental area with non-Bt soybeans in February 2020. The area is located in the Brazilian central-west region (S – 15.908236, W – 48.033878) in Brasília, Federal District, and was selected for the field trials with *M. rileyi* (see following subsection). During dissection, the corpses were filled with resting spores as described by Harper and Carner [7]. Dead larvae showing signs of disease were collected, and fungal structures (resting spores, hyphal bodies, and conidia) were separated from cadavers using syringe needles under a stereoscope. DNA was extracted from resting spores or hyphal bodies using an extraction kit (PureLink Genomic DNA Mini Kit, Invitrogen), and species identification was performed using partial sequences of the genes *LSU*, *SSU*, and *rpb2* [26]. Multiple sequence alignments of these three genes were assembled and compared to sequences available in GenBank. A partial sequence of the internal transcribed spacer region (ITS) was also obtained but was not included in the analysis due to the lack of reference sequences for this DNA barcode. Alignment analysis was carried out under the maximum likelihood criterion using W-IQTree software [27], and bootstrap support (ML) values were provided. Purified DNA and fresh resting spores recovered from infected cadavers were stored in Tris–EDTA buffer solution (pH 8.0) and glycerol (10%) at – 80 °C, respectively.

Efficacy of *Metarhizium rileyi* Formulations and the Impact of Chemical Fungicides on the Occurrence of Caterpillar-Pathogenic Fungi in Soybean

Field trials were conducted in a soybean field (ca. 3.5 ha) in the reproductive growth stage R3 (initial pod development and ca. 50–55 cm tall) at the beginning of February for two subsequent years (2020 and 2021). In both years, plots of 100 m^2 were delimited and separated by 20-m-wide strips of untreated plants. The experiments had a completely randomized design with four replicates (plots) per treatment. Plots were selected next to the area of occurrence of *P. gammae*, and the impact of chemical fungicide spray on this fungal species was also investigated. In both experiments, the treatments were applied to the plots using a CO_2-powered backpack sprayer with nozzles affixed to a horizontal bar and directed down to the plants. The sprayer homogenously delivered a volume of 200 L ha^{-1} at 35 lb pol^{-2}. In 2020, treatments consisted of OD, WG, WP formulations, and an NF preparation suspended in water at a concentration of 1×10^7 conidia mL^{-1} (2×10^{12} conidia ha^{-1}). Untreated plots were used as the negative controls. In 2021, plots were previously treated with azoxystrobin + benzovindiflupyr at the same concentration used in the laboratory experiments. After 2 h, the plots were sprayed with the OD formulation or NF *M. rileyi* preparation at a concentration of 2×10^7 conidia mL^{-1} (4×10^{12} conidia ha^{-1}). The OD formulation and NF preparation were also applied to plots without fungicide

treatment. Untreated plots and plots treated with only fungicide were used as controls.

Insects were sampled in each plot by shaking soybean plants over a cloth (0.5 × 1 m) between two rows to dislodge caterpillars from leaves. All caterpillars found on the cloth were immediately transferred to containers containing soybean leaves. Insects were collected before treatment and 4 days post-spraying in both years, and after 14 days and 24 days in 2021. Insects from each plot were counted, individualized, and maintained on an artificial diet until the pupal stage. Insect mortality caused by fungal infection was evaluated, and the cause of death was confirmed microscopically by observing the reproductive structures according to Humber [28]. The number of parasitoids (wasps and tachinids) that emerged from the larvae and pupae was also evaluated.

Statistical Analyses

Laboratory and field experiment analyses were performed using R Statistical Software [29]. Survival analyses (package "survival") were used to estimate the mean survival times (ST_{50}) of larvae exposed to the different formulations in the laboratory, and the survival curves were compared using Cox proportional hazard regression (Wald test $p < 0.05$). The percentages of germinated conidia, insects killed by *M. rileyi* in the lab, and fungal pathogens and parasitoids in the field experiments were fitted to a generalized linear model (GLM) with binomial distribution (logit-link function). Model selection was performed to choose the best model to fit proportional data using the package "hnp" with overdispersion considered [30]. Mean values were statistically separated by Tukey's HSD test at $p < 0.05$ (function "glht" in package "multcomp").

Results

Bioassays of the *Metarhizium rileyi* Formulations Against *Chrysodeixis includens* Larvae

Differences in survival curves in the bioassays with *C. includens* were found among treatments ($\chi 2 = 68.37$; df = 4; $p < 0.001$). Strain CG1153 formulated in OD had the lowest ST_{50} value, differing from the other formulations and the control treatment, despite its similarity to the NF preparation. Confirmed mortalities caused by all the treatments with *M. rileyi* were similar ($\chi 2 = 10.26$; df = 3, 20; $p = 0.129$). In fact, between 61.2% (WG) and 77.2% (OD) of treated *C. includens* larvae were successfully colonized by the fungus (Table 1).

In Vitro and In Vivo Effects of Chemical Fungicides on Formulated and Unformulated *Metarhizium rileyi* Conidia

Both chemical fungicides had a remarkable negative impact on the viability of NF and formulated conidia of *M. rileyi*, killing all cells after exposure to solid culture media. The germination rates of NF and formulated conidia that were not exposed to fungicides were found to significantly differ ($\chi 2 = 35.88$; df = 3, 12; $p = 0.002$). The germination percentages ranged from 80 to 90%; however, the germination percentage of WG was statistically lower than that of the other formulations (OD and WP) and the NF preparation (Fig. 1).

Table 1 Mean survival times (ST_{50}) for *Chrysodeixis includens* third-instar larvae exposed to formulated and unformulated *Metarhizium rileyi* conidia at 26 ± 1 °C, RH > 90%, and complete darkness

Treatments	ST_{50} (in days)[1]		Confidence interval		Confirmed mortality (%)[3]	
			Lower	Upper		
OD	7.68	a	7.57	7.79	77.2 ± 3.01	a
WP	7.93	b	7.82	8.04	71.9 ± 5.59	a
WG	8.22	c	8.10	8.34	61.2 ± 5.51	a
NF	7.76	ab	7.64	7.88	71.4 ± 3.26	a
C	ND[2]	d	ND	ND	-*	

OD oil dispersion, *WP* wettable powder, WG wettable granules, *NF* non-formulated conidia, sprayed at a concentration of 1×10^7 conidia mL^{-1} of water (0.05% Tween 80), *C* negative control (sprayed only with 0.05% Tween 80)

[1]Time-response values followed by different letters within host species are significantly different by Cox proportional hazard regression ($p < 0.05$)

[2]ND—not determined as insect mortality was < 1.5% in the control treatment

[3]Mortality (± SEM) of insects with mummified bodies 10 days after treatment due to *M. rileyi* colonization. Values followed by different letters are significantly different according to Tukey's honestly significant difference test at $p < 0.05$

*No mortality caused by *M. rileyi* was observed; therefore, the control treatment was not included in the statistical analysis

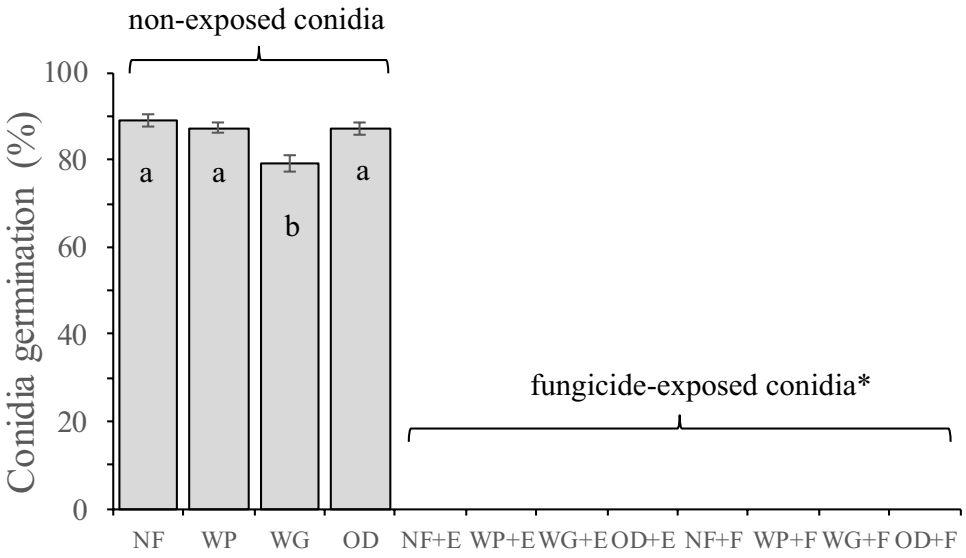

Fig. 1 Germination (%) on agar-medium of formulated (OD—oil dispersion, WP—wettable powder, WG—wettable granules) and unformulated (NF) conidia of *Metarhizium rileyi* exposed or not exposed to the chemical fungicides, azoxystrobin + benzovindiflupyr WG [100 g.100 L^{-1}] (E) and trifloxistrobina + prothioconazole SC [400 g.100 $L.^{-1}$] (F), after 48 h of incubation at 26 ± 1 °C. Values followed by different letters are significantly different by Tukey's HSD test at $p < 0.05$. *No germination was observed; therefore, treatments were not included in the statistical analysis

Larval mortality in the laboratory was also affected when chemical fungicides were applied to the soybean leaves before fungal treatment ($\chi 2 = 279.47$; df = 8, 63; $p < 0.001$). The percentage of dead larvae decreased from 61.7% for OD-formulated conidia, which were fungicide-free, to less than 22% in fungicide-treated leaves. The same trend was observed for NF conidia, as larval mortality reached 45.3% without the use of fungicides and was less than 15% in leaves previously treated with fungicides (Fig. 2). The effect of fungicides on larval mortality was minimal (< 5.5%), similar to that of untreated larvae, and none of the dead insects showed signs of fungal infection. The percentage of fungus-infected larvae (confirmed mortality) also differed among treatments ($\chi 2 = 159.34$; df = 5, 42; $p < 0.001$), with fungicides affecting *M. rileyi* under laboratory conditions (Fig. 2). The reduction in cadaver colonization by unformulated conidia exposed to the fungicides, azoxystrobin + benzovindiflupyr (NF + E) and trifloxistrobina + prothioconazole (NF + F), in relation to fungicide-free treatments was 38.9% and 61.6%, respectively. In relation to the OD-formulated conidia, this

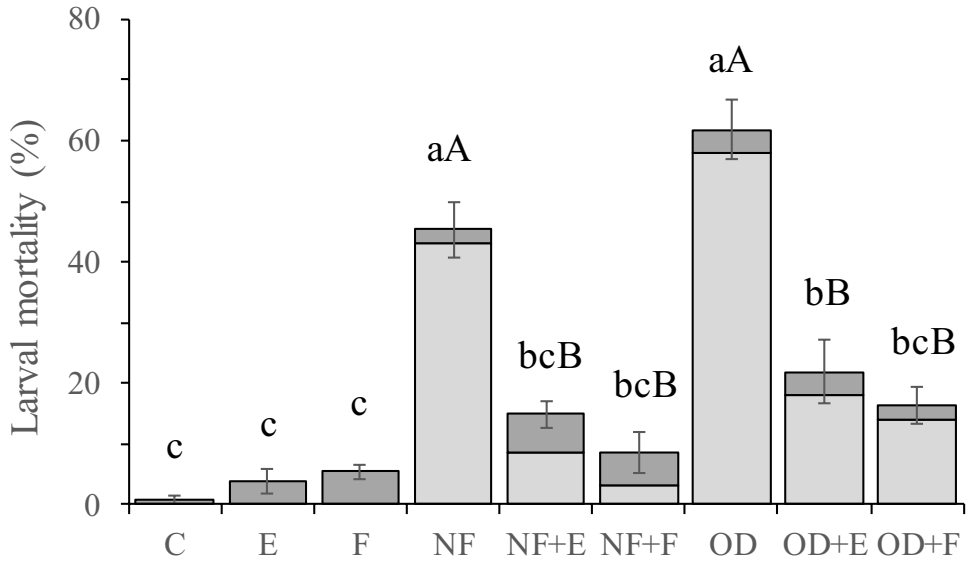

Fig. 2 Mortality (%) of *Chrysodeixis includens* larvae 10 days after spraying formulated (OD) and unformulated (NF) *Metarhizium rileyi* conidia (6×10^3 conidia cm^{-2}) on soybean leaf discs treated or not treated with the chemical fungicides, azoxystrobin + benzovindiflupyr WG [100 g.100 L^{-1}] (E) and trifloxistrobina + prothioconazole SC (F) [400 g.100 $L.^{-1}$] (F), at 26 ± 1 °C, RH > 90%, and complete darkness; C—negative control (sprayed only with the oil-in-water carrier at 1%). Values followed by the same lower (total mortality; full bars) and upper (confirmed mortality; light gray bars) case letters are not significantly different ($p < 0.05$)

reduction was only 12.3% and 8.5% for OD + E and OD + F, respectively.

Natural Occurrence of Fungal Entomopathogens in a Soybean Field and Species Identification

Cadavers of *C. includens* were found attached to soybean leaves in February 2020, showing typical signs of an entomophthotalean infection. Soybean looper caterpillars of different instars collected and kept in the laboratory developed both conidial and resting spore forms of the disease. In the first case, the larval bodies became shriveled, a light-yellow mat of vegetative cells developed, and conidia were produced. The resting spore form, which was mainly observed on later-instar larvae, resulted in a black leathery cadaver filled with spores. The incidence of the disease in *C. includens* reached 23.4% of the sampled population ($n = 303$) and three of the four cadavers developed resting spores. The velvetbean caterpillar, *A. gemmatalis*, was collected (approximately 15% of collected larvae; $n = 55$); however, none of the larvae developed this particular disease.

Based on phylogenetic analysis of the combined partial *LSU*, *SSU*, and *rpb2* gene datasets (*LSU* = 970, *SSU* = 1104, and *rpb2* = 1259 base pairs), the naturally occurring fungus was placed within Entomophthoraceae. The specimen clearly clustered with other species in this family (Supplementary Information Fig. 5) and was closely related to other isolates within the *Furia-Pandora* clade. Based on two distinct physiological forms, host specificity, and multigene analysis, this fungus was identified as *P. gammae* (= *Entomophthora gammae*). Partial sequences from the *ITS*, *LSU*, *SSU*, and *rpb2* genes were deposited in GenBank under accession numbers OM732267, OM732269, OM732268, and OM850691, respectively.

Efficacy of the *Metarhizium rileyi* Formulations and Impact of Chemical Fungicides on the Occurrence of Caterpillar-Pathogenic Fungi in Soybean

In 2020, a mixed infestation of *A. gemmatalis* and *C. includens* was observed in soybean fields. Soybean looper was predominant during the entire experimental period, despite a decrease in its percentage in relation to the velvetbean caterpillar from 80.2% on the first sampling date (immediately prior to spraying) to 73.8% on the second (4 days post-spraying). Few armyworm (*Spodoptera* sp.) larvae were also found (< 5.5%), but being not included in the analyses. Natural infection by *M. rileyi* was very low before treatment (less than 0.6% of the population), whereas *P. gammae* and parasitoids were the main cause of natural *C. includens* mortality (Fig. 3). The evaluation date ($\chi 2 = 2.83$; df = 1, 34; $p = 0.520$) and formulations type ($\chi 2 = 4.33$; df = 4, 35; $p = 0.146$) did not interfere with the natural larval infection rates by *P. gammae*, and no significant interaction treatment time was seen ($\chi 2 = 8.44$; df = 4, 30; $p = 0.178$) (Supplementary Information Table 2). Similarly, an interaction between time and treatment was not observed in parasitoid populations ($\chi 2 = 0.72$; df = 4, 30; $p = 0.949$). The formulations had no effect on parasitism ($\chi 2 = 12.94$; df = 4, 35; $p = 0.115$), and a decrease in the number of parasitoids in time was found ($\chi 2 = 52.14$; df = 1, 34; $p < 0.001$). Parasitism was greater in the preliminary evaluation than 4 days after spraying (Supplementary Information Table 4). Unformulated *M. rileyi* conidia and all formulations applied to soybean plants induced significant infection rates in the caterpillar population ($\chi 2 = 30.39$; df = 4, 15; $p < 0.001$). Four days after spraying, the number of infected larvae in treated plots was greater compared to untreated plots; however, differences among fungal treatments in performance were not observed (Fig. 3, Supplementary Information Table 2.

In 2021, no background *M. rileyi* infection was observed. However, the fungus, *P. gammae*, persisted in the field, and approximately one of seven *C. includens* larvae died from this fungus. Parasitoids caused 36% larval mortality immediately before the beginning of the experiment. A mixed infestation of *A. gemmatalis* and *C. includens* was also present in 2021, and the percentage of soybean looper in relation to the velvetbean caterpillar in the population decreased over time, from 76.1% before treatment to 41.8% after 24 days (Fig. 4). No differences were found in the occurrence of *P. gammae* ($\chi 2 = 3.62$; df = 5, 90; $p = 0.605$) and parasitoids among treatments ($\chi 2 = 5.28$; df = 5, 42; $p = 0.383$); however, a significant reduction in the number of parasitoids before and 24 days after treatment was observed ($\chi 2 = 82.80$; df = 1, 41; $p < 0.001$) (Supplementary Information Tables 3 and 4). There was a significant interaction between the *M. rileyi* treatment and evaluation date ($\chi 2 = 20.63$; df = 6, 39; $p = 0.002$). A single application of OD-formulated and unformulated conidia induced mortality levels of 10–36% and 37–47% in insects collected after 4 and 24 days, respectively, without significant negative effects of the fungicide on larval infection. Larval mortality caused by the fungus in OD-formulated conidia increased over time compared to that in NF treatment (Fig. 4, Supplementary Information Table 3).

Discussion

Chemical fungicides have been reported as obstacles to the broader adoption of fungal-based biopesticides, including the caterpillar killer fungus, *M. rileyi*, and their persistence in the field [3, 12, 13]. In our study, some chemical fungicides deployed in soybean crops to control the rust, *Phakopsora pachyrhizi*, were found to have a remarkable

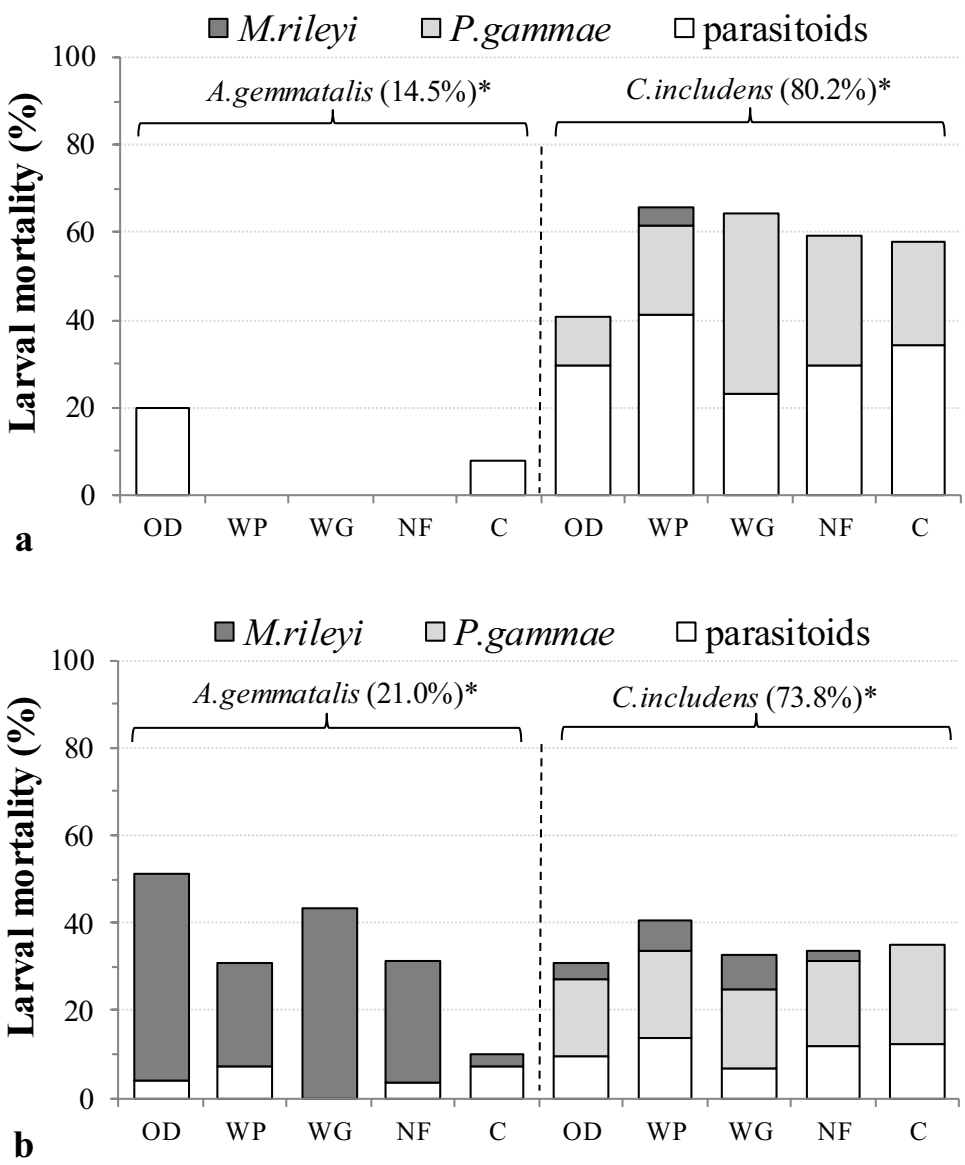

Fig. 3 Mortality (%) of *Anticarsia gemmatalis* and *Chrysodeixis includens* larvae before (**a**) and 4 d after spraying (**b**) formulated and unformulated *Metarhizium rileyi* conidia in a soybean field (Jan.–Feb. 2020). *Average percentage of each lepitopteran species in the collected sample. OD—oil dispersion, WP—wettable powder, WG—wettable granules, NF—non-formulated conidia, sprayed at a dose of 1×10^{12} conidia ha^{-1}; C—negative control (unsprayed)

negative impact on conidial germinability and host infectivity by the fungus, *M. rileyi*, under laboratory conditions. However, a single application of these fungicides to soybean plants in the field, followed by spraying of formulated and unformulated *M. rileyi* conidia, did not have a significant negative effect on the infection rates of *A. gemmatalis* and *C. includens* over time.

In general, the low-cost formulations used in our laboratory experiments were effective at infecting *C. includens* larvae. The OD formulation had some advantages, such as increasing the rate of the infection process and reducing the time to kill the host (lower ST_{50}), despite no significant difference in the reduction in survival time relative to that observed for unformulated conidia. The advantages of oil-based formulations in enhancing the infectivity of the selected strains of *M. rileyi* [9, 19, 20, 23] and reducing damage to plants [22] have been reported in other studies. Emulsifiable oils facilitate biopesticide manipulation by the end user as an alternative to formulate highly hydrophobic dry conidia of *M. rileyi*. The WG formulation also made handling easier and provided good wettability, but resulted in a greater ST_{50}. The lower performance of WG might be related to conidial vigor, which was affected by the washing procedure used to prepare the formula [31]. This effect was more evident in in vitro experiments.

In laboratory experiments, all tested formulations had little impact on the protection of conidia against the negative effects of chemical fungicides. This harmful effect was clearly observed when the conidia were directly exposed to fungicides applied to the medium surface. Under these conditions, continuous contact of the fungus with field-recommended doses of both fungicides completely killed

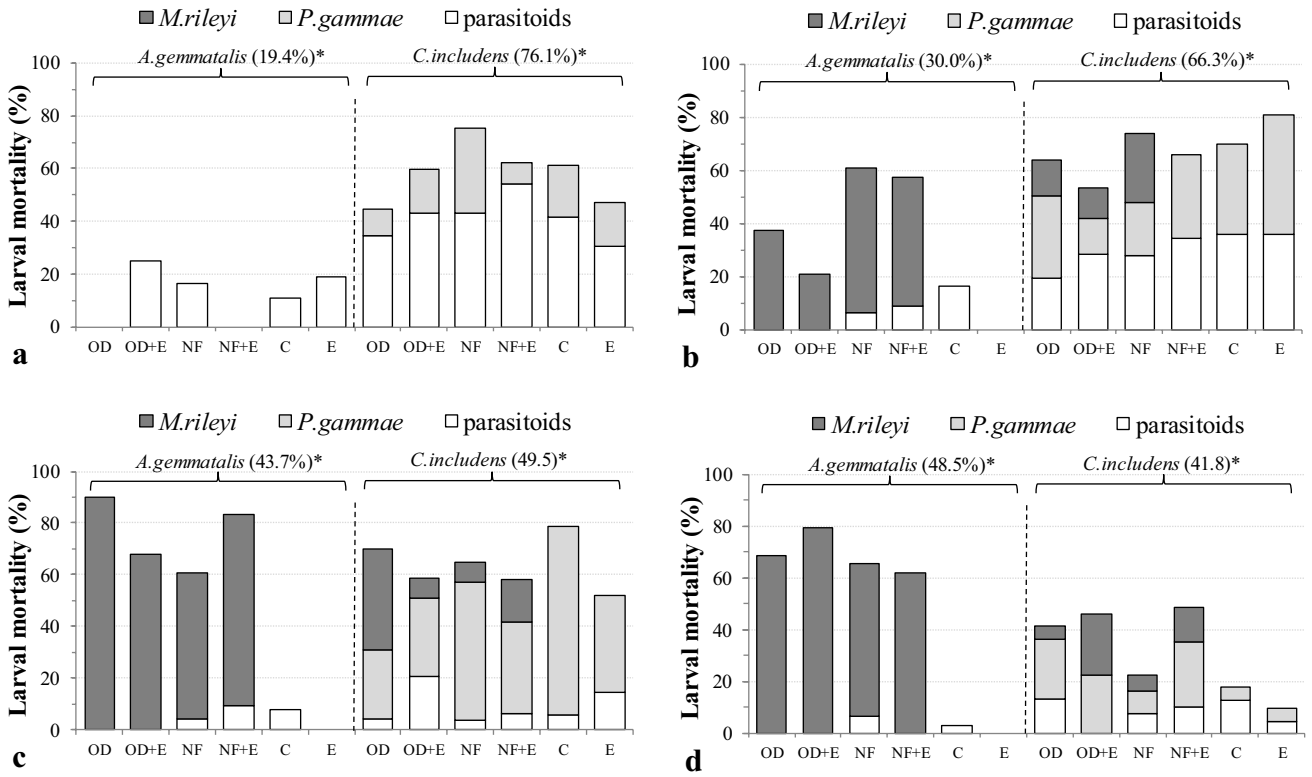

Fig. 4 Mortality (%) of *Anticarsia gemmatalis* and *Chrysodeixis includens* larvae before (**a**) and 4 days (**b**), 14 days (**c**), and 24 days after spraying (**d**) formulated and unformulated *Metarhizium rileyi* conidia in a soybean field (Jan.–Feb. 2021). *Average percentage of each lepidopteran species in the collected sample. OD—oil dispersion and NF—non-formulated conidia, sprayed at a dose of 2×10^{12} conidia ha^{-1}; E—chemical fungicide (azoxystrobin + benzovindiflupyr WG—200 $g.ha^{-.1}$), C—negative control (unsprayed)

the infective cells. The proportion of cadavers colonized by *M. rileyi* in the lab experiments was also reduced by treatments containing fungicide residues compared to fungicide-free treatments. This reduction in cadaver colonization was observed for both unformulated and OD-formulated conidia exposed to fungicides, but was apparently lower in the latter, suggesting that some protection was afforded by emulsifiable oils. Nonetheless, the successful infection of a considerable proportion of fungal-treated insects exposed to leaves contaminated with fungicide indicates that the adverse effects observed in vitro are not necessarily replicated under field conditions, as previously suggested by Jaronski [32]. This researcher suggested that conidia may become dormant on leaf surfaces until they contact the insect cuticle, and some chemical pesticides are readily absorbed by leaves following application, rendering a very short exposure of the fungus to fungicide residues.

The percentage of *A. gemmatalis* caterpillars infected by *M. rileyi* in the 2020 field trial was higher than *C. includens*, with the former occurring in lower numbers. In fact, *A. gemmatalis* is considerably more susceptible to this strain than *C. includens* [9], and the great natural mortality of *C. includens* caused by parasitoids and *P. gammae* reduced the number of this host in the field and the chances of a higher *M. rileyi* infection rate. The entomophthoralean, *P. gammae*, seems to be restricted to American countries and has been previously reported in members of the family Plusiinae in the southern USA [7, 8, 10, 33–35], Mexico [36, 37], Argentina, and Brazil [4], mainly inducing infection rates in the 25–30% range, as observed in our study. Although abundant literature from 1970 to 1990 provides an in-depth description of the morphological and epizootiological characteristics of *P. gammae*, molecular data on this species have not been generated to date. According to Gryganskyi et al. [26], the resolution of species delimitation within the fungal subfamily Erynioideae still requires more complete sampling and more gene sequences for taxa included in this group. The sequences obtained from *P. gammae* in our study are the first to be available in GenBank.

In 2020, the occurrence of disease caused by *M. rileyi* in populations of soybean caterpillars was insufficient to produce primary infection sites at the beginning of the following year, as infected larvae were not detected before the second sampling date. As observed in 2020, the percentage of *A. gemmatalis* infected by *M. rileyi* in 2021 was higher than that of *C. includes*, and the percentage of larvae killed

by this fungus was also higher than that in the previous year, when the dose of conidia applied per hectare was lower. On the other hand, during 2021, *P. gammae* persisted in the area and repeated epizootics in the *C. includens* population. Lower infection rates were expected in fungicide-treated plots, as the fungus was applied only few hours later. However, the negative impact observed in the laboratory bioassay was not observed in the field, as the fungus could infect part of the population (readings at 4th day post-spraying) and generated secondary infections (readings at 14th and 24th days post-spraying) in both fungicide-treated and fungicide-free plots.

Fungicides may affect other entomopathogenic fungi besides *M. rileyi*. For instance, the application of fungicides significantly lowered the prevalence of entomophthoralean diseases in aphid populations in cotton fields [38, 39] and soybean [40]. In 2021, we verified that the well-established natural epizootics of *P. gammae* on *C. includens* might not be affected by a single spray of the soybean rust fungicide (azoxystrobin + benzovindiflupyr). According to Clifton et al. [41], fungicides and herbicides applied to conventional crop systems may not have a significant effect on entomopathogenic fungi in the soil environment, where resting spores and other fungal propagules may persist. Livingston et al. [42] also showed that other fungicide groups failed to suppress epizootics of *P. gammae* on *C. includens* populations. Likewise, *M. rileyi* inhibition by non-selective fungicides (benomyl or difenoconazole) was insufficient to prevent outbreaks of this fungus on these caterpillars. Further, on most sampling dates, the occurrence of *A. gemmatalis* in soybean fields was not observed [14]. On the other hand, Stansly and Orellana [43] reported that *M. rileyi* inhibition by benomyl and chlorothalonil caused significantly higher populations of *A. gemmatalis* and *C. includens*. Although many factors appear to be involved in the natural occurrence of *M. rileyi* in lepidopterous populations following the application of chemical fungicides, our study suggests that epizootics following the application of *M. rileyi*-based pesticides are less affected by these chemicals.

Biocontrol agents found in soybean fields (parasitoids, tachinids, and *P. gammae*) and the sprayed fungus, *M. rileyi*, are lepidopteran-specific natural enemies that may compete for the same hosts. In both years of the field trials, these natural enemies were complementary to caterpillar control. The high number of *C. includens* larvae at the beginning of the infestation (ca. 75% of sampled lepidopterans) was readily regulated by the plusiinae-specific pathogen, *P. gammae*. After a single spray, *M. rileyi* CG1153 formed primary infection sites on an increasing population of *A. gemmatalis*, a host highly susceptible to this strain, and controlled a portion of the *C. includes* populations. The prominent role of naturally occurring parasitoids (flies in the family Tachnidae and, especially, wasps) in the regulation of *Spodoptera frugiperda* in corn was recently shown by Faria et al. [23]. The present work also highlighted the importance of conservation biological control in soybean crops, since, as in the previous study, parasitism rates in both years exceeded 40–50% in some cases.

In summary, a single foliar application of a non-selective fungicide might be harmless to both *M. rileyi* and *P. gammae* in soybean fields. However, based on laboratory bioassays, the higher the degree of exposure, the greater the effect on fungal entomopathogens. In the absence of selective fungicides, reducing the frequency of application and appropriate timing may be possible alternatives to mitigate any potential negative impact on fungal entomopathogens (and possibly parasitoids) under field conditions.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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