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Comparative effectiveness of *Metarhizium rileyi*, novaluron, and glyphosate on immune system, development, and redox metabolism of *Anticarsia gemmatalis*

Eficácia comparativa de *Metarhizium rileyi*, novaluron e glifosato no sistema imunológico, desenvolvimento e metabolismo redox de *Anticarsia gemmatalis*

Efectividad comparativa de *Metarhizium rileyi*, novaluron y glifosato en el sistema inmunológico, el desarrollo y el metabolismo redox de *Anticarsia gemmatalis*

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Abstract
Anticarsia gemmatalis is one of the most important pests in world soybean crop. The most common intervention is the application of agrochemicals, such as novaluron and glyphosate. Among biological control agents, much attention has been drawn to entomopathogenic fungi, as *Metarhizium rileyi*. Here, we examined the changes that occur in the immune system (total and differential hemocyte count), secondary effects (caterpillar morphology), and oxidative metabolism after the caterpillars were exposed to *M. rileyi*, novaluron or glyphosate. *M. rileyi* was able to induce changes in the width, length, and weight of *A. gemmatalis* pupae, along with an increased in the number of defense cells. Novaluron prompt changes the insect's immunity, and glyphosate caused milder immunological effects. However, it caused significant secondary effects including malformations in pupae and adults, and an increase in nitric oxide (NO) levels. Mortality observed when treating insects with novaluron and malformations due to glyphosate treatments did not occur due to oxidative stress. However, when insects were exposed to *M. rileyi*, we verified significantly increased levels of NO and concluded that these insects died due to oxidative stress. Our data provide evidence that contributes to better understanding the mechanism of herbicide-fungus interaction in the management of *Anticarsia gemmatalis.*

Keywords: Agrochemical; Pest control; Entomopathogenic fungus; Oxidative stress; Soybean; Hemocytes.

Resumo

Anticarsia gemmatalis é uma das principais pragas da cultura da soja. A intervenção mais comum é a aplicação de agroquímicos, como novaluron e glifosato. Dentre os agentes de controle biológico, muita atenção tem sido dada aos fungos entomopatogênicos, como *Metarhizium rileyi*. Neste trabalho, avaliamos as mudanças que ocorrem no sistema imunológico (contagem total e diferencial de hemócitos), efeitos secundários (morfologia da lagarta) e metabolismo redox após as lagartas serem expostas a *M. rileyi*, novaluron ou glifosato. *M. rileyi* foi capaz de induzir mudanças na largura, comprimento e peso das pupas de *A. gemmatalis*, juntamente com um aumento no número de células de defesa. Novaluron altera a imunidade do inseto e o glifosato causou efeitos imunológicos mais suaves. No entanto, causou efeitos secundários significativos, incluindo malformações em pupas e adultos, e um aumento nos níveis de óxido nítrico (NO). A mortalidade observada no tratamento de insetos com novaluron e malformações devido ao tratamento com glifosato não ocorreu devido ao estresse oxidativo. No entanto, quando os insetos foram expostos a *M. rileyi*, verificamos níveis significativamente aumentados de NO e concluímos que esses insetos morreram devido ao estresse oxidativo. Nossos dados fornecem evidências que contribuem para o melhor entendimento do mecanismo de interação herbicida-fungo no manejo de *Anticarsia gemmatalis.*

Palavras-chave: Agroquímico; Controle de pragas; Fungo entomopatogênico; Estresse oxidativo; Soja; Hemócitos.

Resumen

Anticarsia gemmatalis es una de las plagas más importantes del cultivo mundial de la soya. La intervención más común es la aplicación de agroquímicos, tales como novaluron y glifosato. Entre los agentes de control biológico, se ha prestado mucha atención a los hongos entomopatógenos, como *Metarhizium rileyi*. Aquí, examinamos los cambios que ocurren en el sistema inmunológico (recuento de hemocitos total y diferencial), los efectos secundarios (morfología de la oruga) y el metabolismo oxidativo después de que las orugas fueron expuestas a *M. rileyi*, novaluron o glifosato. *M. rileyi* pudo inducir cambios en el ancho, largo y peso de las pupas de *A. gemmatalis*, junto con un aumento en el número de células de defensa. Novaluron cambia rápidamente la inmunidad del insecto y el glifosato provoca efectos inmunológicos más leves. Sin embargo, causó efectos secundarios importantes, incluidas malformaciones en pupas y adultos, y un aumento en los niveles de óxido nítrico (NO). La mortalidad observada al tratar insectos con novaluron y las malformaciones debidas a los tratamientos con glifosato no ocurrió por estrés oxidativo. Sin embargo, cuando los insectos estuvieron expuestos a *M. rileyi*, verificamos niveles significativamente mayores y concluimos que estos insectos murieron debido al estrés oxidativo. Nuestros datos proporcionan evidencia que contribuye a comprender mejor el mecanismo de interacción herbicida-hongo en el manejo de *Anticarsia gemmatalis.*

Palabras clave: Agroquímico; Control de plagas; Hongo entomopatógeno; Estrés oxidativo; Soya; Hemocitos.

1. Introduction

Soybean is one of the most produced and consumed cereals in the world (da Silva Júnior et al., 2020), and Brazil is one of the main producers and exporters of the soy complex (bran, oil, and grain), with numerous possibilities for expansion (Deagro, 2016). In the 2019/2020 harvest, world production was around 339 million tons (Fiesp, 2020), but even in the face of advances in the cropping system, pests remain a serious problem, requiring the constant use of insecticides.

The velvetbean caterpillar *Anticarsia gemmatalis* (Hübner; Lepidoptera: Noctuidae) is the main soybean pest in the Americas (Haase et al., 2015; Kogan & Turnipseed, 1987). It feeds on leaves, and in some cases, can cause 100% defoliation, consequently, impairing the filling of the pods (Moscardi et al., 2012). *A. gemmatalis* can cause an estimated loss of 15.6% of crop yield (Kreyci & Menten, 2013) and is one of the main causes for economic losses (Haase et al., 2015). In the face of such aspects, the design and develop of new strategies for their management is crucial.

The success in implementing effective integrated pest management programs depends on the understanding of the action of agrochemicals and biological control agents on the insects and their compatibility. The most common form of management remains the application of insecticides (Castro et al., 2019), one of which is Rimon SUPRA®, whose active principle is novaluron (World Health Organization, 2004). Novaluron is an insect growth regulator used in crops. Moreover, an herbicide usually applied in soybean management is Roundup WG®, whose active principle is glyphosate. This nonselective herbicide (ANVISA, 2018) can also target non-target organisms, affecting the biological balance of species (Monserrat et al., 2007; Schneider et al., 2009). Besides the use of agrochemicals, adjuvant strategies involve biological control agents.

Biological control is part of Integrated Pest Management (IPM), which combines methods such as biological, physical, mechanical, and chemical, that can minimize economic damage (Senar, 2018). Moreover, biological control is able to reduce environmental impacts, due to the high specificity and selectivity exhibited by the bioagents (Alves, 1998). One organism that can be used in these alternative systems is entomopathogenic fungi, such as the *Metarhizium rileyi* (Farlow) Kepler, SA Rehner and Humber.

M. rileyi usually infects the caterpillar in two major distinguishable ways: by the tegument and the ingestion. The main mechanism of action displayed by the fungus includes the production of mycotoxins blocking the digestive system by the production of blastospores that are able to replicate intensely in the hemolymph, thus colonizing the entire insect (Alves, 1998; Lopez-Lastra and Boucias, 1994; Wang & St. Leger, 2006). *M. rileyi* can infect more than 60 host species (Fronza et al., 2017), mostly lepidopterans (Song et al., 2017), including *A. gemmatalis*.

The defense system of insects against chemical or biological agents includes several mechanisms. First are the physical barriers, which include the cuticle, the peritrophic membrane of the intestine, and the tracheal system. Second, cellular immunity (Gonzalez et al., 2013; Tomás-Barberán et al., 2014) and humoral immunity (Strand, 2008; Tanaka & Yamakawa, 2011) are activated as well as the antioxidant defense system. The latter includes a group of detoxifying enzymes such as superoxide dismutase and catalase. Previous studies have demonstrated that agrochemicals such as glyphosate (Monserrat et al., 2007; Sies, 1993) and even some natural extracts (Branco et al., 2016, 2014) are able to cause an imbalance between the production of ROS and the antioxidant system, inducing entomotoxic effects by increasing oxidative stress. Oxidative damage to biomolecules, such as lipid peroxidation and protein modifications, can lead to cell death (Cobb & Cole, 2015). Moreover, increase in the production of nitric oxide (•NO) and its derivatives can also contribute to the nitrosylation of proteins and lipids (O'Donnell et al., 1999). •NO is an important molecule involved with the insect's humoral response (Faraldo et al., 2005), activating plasmocytes (PL), granulocytes (GR), oenocytes (OE), spherulocytes (SP), adipohemocytes (AD), and prohemocytes (PR) (Gonzalez et al., 2013; Strand, 2008). The modulation of these cells in response to the exposure to agrochemicals and biocontrol agents remains to be elucidated.

Considering the compelling evidence, the present study analyzed the immune system, biological parameters, and oxidative stress levels of *A. gemmatalis* when infected by the entomopathogenic fungus *M. rileyi* and when fed with novaluron and glyphosate. The data presented here will help to better clarify host-pathogen interactions, providing valuable information for planning new biological control strategies.

2. Methodology

2.1 Insects

The *Anticarsia gemmatalis* caterpillars were reared on an artificial diet (Greenne et al., 1976), and kept in the Laboratory of Pest Control - Institute of Biotechnology, University of Caxias do Sul at 26 °C ± 1 and 70% ± 1 relative humidity (RH).

2.2 Chemical Insecticide

Rimon SUPRA®, a commercial novaluron-based product is registered with the Brazilian Ministry of Agriculture, Livestock, and Food Supply, under number 14511, with the company Adama Brazil holding the registration (Agrofit, 2015). It is composed of 100 g/L of novaluron and 1020 g/L of inert ingredients. Herbicide Roundup WG®, a commercial product based on glyphosate, is manufactured by Monsanto and registered with the Brazilian Ministry of Agriculture, Livestock, and Food Supply under number 2094 (Agrofit, 2015). Its active ingredient is glyphosate ammonium salt 792.5 g/kg (720 g/kg equivalent of N-phosphomethy) glycine (Glyphosate) along with 207.5 g/kg of inert ingredients.

2.3 Entomopathogenic fungus

Fungal isolate Sa86101 of *M. rileyi* (isolated from *A. gemmatalis* collected in Sarandi, RS, Brazil) was maintained in the collection of the Laboratory of Pest Control of the Institute of Biotechnology, University of Caxias do Sul, Brazil.

2.4 Bioassays and treatments

The bioassays were conducted in a climatized room (25 ± 1 °C; 70 ± % RH) (Silva et al., 2007). The herbicide glyphosate (6.3 and 12.5 µg/100 ml) and the insecticide novaluron (6.3 and 12.5 µL/100 ml) were added homogeneously into the artificial diet. The doses selected for the tests were those that allowed the survival of the insects to enable collection of hemolymphs. The bioassays with glyphosate were done with third instar caterpillars and the bioassays with novaluron with fifth instar caterpillars. The *M. rileyi* entomopathogen bioassays were performed with suspensions of 1.10^7 and 1.10^9 conidia/ml distributed on filter paper in Petri dishes in which third instar caterpillars were placed. The plates were kept in B.O.D. type incubator at 26 °C; 12 h photophase, and 70 ± 5% RH for 24 h. After this period, the insects were transferred to the artificial diet and kept under the same conditions as the other treatments. The caterpillars were placed individually in 50 mL plastic cups containing an artificial diet, as described by Greenne et al. (1976).

2.5 Evaluation of the immune system response

This evaluation was conducted through identification and quantification of hemocytes using a fluorescence microscope. The assay was performed according to the methodology described by Kwon et al. (2014) using caterpillars from bioassays (10 caterpillars/treatment - 2 repetitions). On the eighth and tenth day after starting bioassays with glyphosate and *M. rileyi*, and third and fifth day with novaluron, 3 µl of each insect hemolymph were collected by abdominal puncture diluted (2:1) in an anticoagulant solution. For the total and the differential hemocyte count (THC and DHC, respectively), the methodology was described by Negreiro et al. (2009), in which fresh hemolymph was diluted 10 times with an anticoagulant solution (0.098 M NaOH, 0.186 M NaCl, 0.017 M EDTA, 0.041 M citric acid) and homogenized. The hemocytes were counted in a Neubauer chamber with optical microscope.

2.6 Evaluation of the secondary effects on caterpillar morphology and metabolism redox

Two hundred seventy caterpillars (thirty/treatment) and a control group were used to evaluate morphological changes (weight, length, and width) in pupal phase. For assessment of oxidative parameters, twenty caterpillars between third and fifth instar were used (glyphosate 12.5 µg/100 ml, novaluron 12.5 µl/100 ml, and *M. rileyi* 1.10^9 conidia/ml). Oxidative damage to lipids and nitric oxide ($^{\bullet}$NO) levels were evaluated between the fifth and twelfth day after the beginning of the treatments. Larvae of each treatment (n = 5 insects per ml) were homogenized in ice-cold 50 mM phosphate potassium buffer containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, and centrifuged at 1,500 × *g* at 4 °C for 5 min. To determine the extent of lipid damage, the assay described by Hermes-Lima and Storey (1995) was employed. Briefly, aliquots (100 mL) of the supernatant were mixed with 100 mL of the color reagent (1% thiobarbituric acid [TBA], 50 mM sodium hydroxide (NaOH), 0.1 mM butylated hydroxytoluene [BHT]) and 50 mL 7% (v/v) phosphoric acid. The mixture was placed in a boiling water bath for 15 min. After cooling, 1.5 mL of n-butanol was added to the mixture followed by centrifugation for 5 min at 1600 × *g*. The absorbance of supernatants was measured at 532 nm, and the results were expressed in nm TMP/mg protein. $^{\bullet}$NO levels were determined as nitrite production, using the Griess reaction method (Green et al., 1981). For the assay, 50 µL of supernatants were reacted with an equal volume of Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4) for 10 min at room temperature, and the absorbance was read at 550 nm. Sodium nitroprusside was used as the standard, and the results were expressed as nmol nitrite/mg protein.

2.7 Evaluation of the sensitivity of the entomopathogenic fungus to glyphosate and novaluron

The tests were performed with the Sa86101 strain of *M. rileyi* according to the methodology described by Botelho & Monteiro (2011). Glyphosate and novaluron were used according to the recommendations of their manufacturers. The recommended doses (RD) of glyphosate and novaluron as well as 50%, 25%, and 12.5% underdoses of the RD were tested. Colony quantification was performed 15 days after inoculation, in a colony counter, marking on the outside of the bottom of the Petri dish. The evaluation of the mycelial growth of the fungus was performed on day 15 after inoculation, measuring, in millimeters, two perpendicular diameters of the colonies. To evaluate the conidial production, a colony was collected from each Petri dish with the aid of a sterile metallic ring 8 mm in diameter, 15 days after incubation. These samples were transferred individually to test tubes containing Tween-80 solution (0.1% v/v). After removing the conidia by vigorous agitation in an electric tube agitator, counting was performed under an optical microscope in a Neubauer chamber, using dilutions of the suspension when necessary.

2.8 Statistical analysis

The Winstat version 1.0 program was employed for statistical analysis. Data were submitted to analysis of variance at 5% probability. In case of significance, a multiple mean comparison was performed by Tukey's test at 5% probability. Except for differential hemocyte counts, the data were transformed by $\sqrt{x} + 0.5$.

3. Results

3.1 Effects of *M. riley,* novaluron, and glyphosate on the caterpillar's total hemocyte count (THC) and differential hemocyte count (DHC)

The results of THC in caterpillars fed a diet with *M. rileyi* (evaluated on the eighth and tenth days after the start of the assays), novaluron and glyphosate (both evaluated on the third and fifth days after the start of the assays) are present in Table 1.

In all treatments, the number of circulating hemocytes in the hemolymph reduced in a time- and dose-dependent manner. For *M. riley*, the greatest reduction (73%) in circulating hemocytes was observed at a dose of 10^9 con/mL, on the tenth day. Specifically, novaluron was able to reduce the number of hemocytes in 43 and 88% on the fifth day at the two doses evaluated. In addition, glyphosate was able to reduce the hemocytes number by 27 to 43% in the same period.

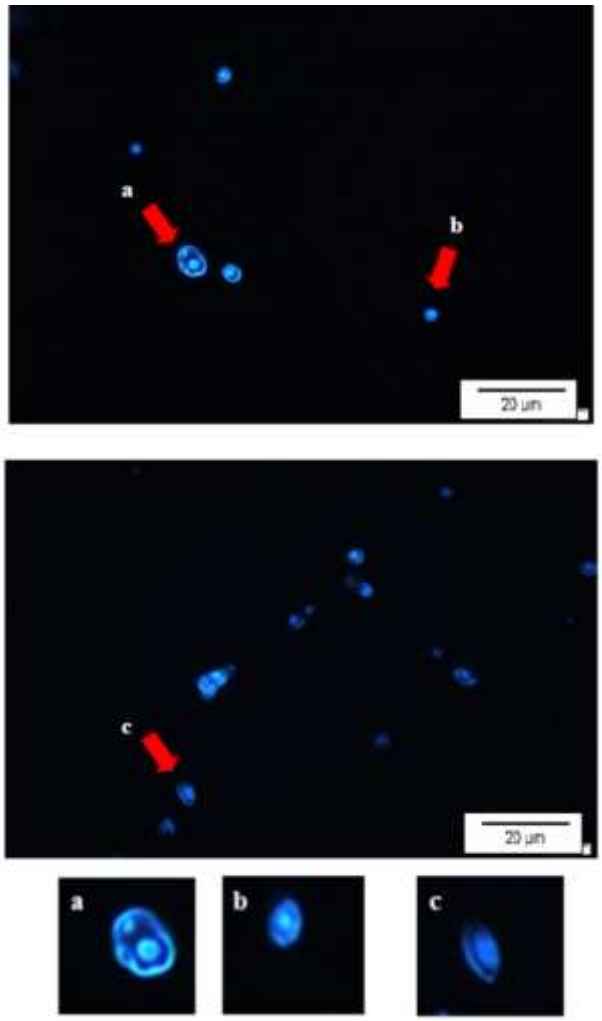
Table 1. Number of total hemocytes of *Anticarsia gemmatalis* (cells/mm³) when infected with the entomopathogenic fungus *M. rileyi* or when fed with novaluron or glyphosate.

Treatment	Dose	3rd day	5th day	8th day	10th day
M. rileyi (Conidia/mL)	Control	-	-	22.700[a]	22.100[a]
	10^7	-	-	17.000[b]	12.400[b]
	10^9	-	-	13.950[c]	6.000[c]
C.V. (%)		-	-	8.19	18.06
Novaluron (µL/100 mL)	Control	21.700[a]	21.700[a]	-	-
	6.3	12.500[b]	4.150[b]	-	-
	12.5	7.450[c]	2.600[c]	-	-
C.V. (%)		5.67	7.29	-	-
Glyphosate (µg/100 mL)	Control	-	-	22.400[a]	22.200[a]
	6.3	-	-	16.350[b]	16.300[b]
	12.5	-	-	13.563[c]	12.850[c]
C.V. (%)		-	-	8.19	18.06

Means followed by different letters in the columns differ statistically by Tukey's test at 5% probability. C.V.= coefficient of variation. Source: Authors.

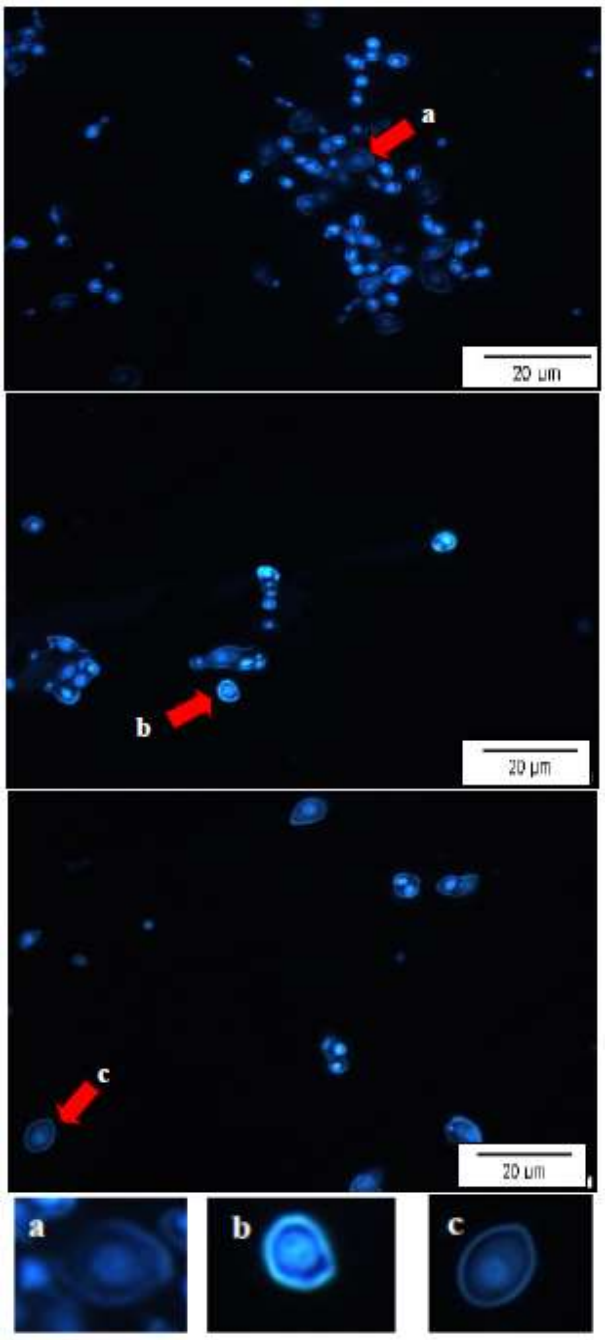
In the hemolymph of *A. gemmatalis*, six categories of hemocytes were identified: oenocytes (OEs) (Figure 1a); prohemocytes (PRs) (Figure 1b); plasmocytes (PLs) (Figure 1c), granulocytes (GR) (Figure 2a), spherulocytes (SPs) (Figure 2b), adipohemocytes (ADs) (Figure 2c).

Figure 1. Hemocyte types in *Anticarsia gemmatalis*: a. Oenocyte; B. Prohemocyte; c. Plasmocyte.



Source: Authors.

Figure 2. Types of *Anticarsia gemmatalis* hemocytes a. Granulocyte; B. Spherulocyte; c. Adipohemocyte.



Source: Authors (2021).

The caterpillars that ate a diet containing novaluron and those that were exposed to the *M. rileyi* exhibited significant changes in the quantity of all hemocytes. Among them, the GRs were the most abundant followed by SPs and PLs. The ADs were numerically less abundant. The decrease in the number of SP was most significant.

In the *M. rileyi* bioassays on the eighth day, the number of PL and GR increased, and the number of circulating cells of the remaining hemocytes decreased. The number of GR exhibited a very significant increase in the dose of 10^9 conidia/mL. Results showed a dose-dependent effect on the number of SP, PR, OE, and AD. On the third day after the start of the novaluron bioassay, a significant difference in the number of PL, SP, and AD was observed between the doses and the control,

with the highest number of cells occurring at the lowest dose. On fifth day, except GR, all other hemocytes decreased in circulating cells in the hemolymph with increasing dose (Table 2).

For the SP and OE evaluated in the glyphosate assays, a statistical difference was observed on the eighth day for the doses in relation to the control. On the 10 days of evaluation, all categories of hemocytes were significantly reduced.

Table 2. Number of differential hemocytes of *Anticarsia gemmatalis* when infected with the entomopathogenic fungus *M. rileyi* or when fed with novaluron or glyphosate.

Treatment	Day	Dose	PL	PR	OE	SP	AD	GR
M. rileyi (Conidia/ml)	8th	Control	18.00^{b}	3.70^{a}	5.30^{a}	34.20^{a}	3.20^{a}	35.60^{b}
		10^7	25.36^{a}	2.60^{a}	5.15^{a}	21.20^{b}	3.15^{a}	41.90^{b}
		10^9	30.25^{a}	2.05^{b}	3.00^{b}	5.30^{c}	2.40^{a}	56.90^{a}
		C. V. (%)	5.43	27.46	17.72	8.38	15.78	3.26
	10th	Control	18.80^{a}	2.60^{a}	5.80^{a}	32.00^{a}	4.40^{a}	36.30^{a}
		10^7	21.41^{a}	2.10^{a}	6.19^{a}	29.07^{a}	3.02^{a}	38.16^{a}
		10^9	13.85^{a}	0.15^{b}	0.50^{b}	0.70^{b}	0.30^{b}	24.50^{a}
		C. V. (%)	39.45	16.81	16.78	8.10	32.38	40.74
Novaluron (µL/100 mL)	3th	Control	15.17^{b}	3.35^{b}	5.46^{a}	31.17^{a}	3.40^{a}	37.39^{a}
		6.3	24.99^{a}	5.02^{a}	4.31^{b}	24.48^{b}	2.11^{b}	42.09^{a}
		12.5	9.20^{c}	4.55^{ab}	5.15^{b}	1.00^{c}	1.10^{c}	42.20^{a}
		C. V. (%)	17.50	16.48	11.78	13.33	17.11	20.97
	5th	Control	15.17^{a}	3.35^{a}	5.46^{a}	31.17^{a}	3.40^{a}	37.39^{ab}
		6.3	15.00^{a}	2.30^{a}	4.15^{a}	18.65^{b}	2.65^{a}	57.15^{a}
		12.5	5.65^{b}	0.60^{b}	1.00^{b}	4.80^{c}	0.30^{b}	27.65^{b}
		C. V. (%)	30.28	19.73	19.08	19.73	17.29	17.29
Glyphosate (µL/100 mL)	8th	Control	17.50^{a}	3.50^{a}	6.80^{a}	31.80^{a}	3.90^{a}	36.70^{a}
		6.3	24.37^{a}	3.04^{a}	4.94^{ab}	19.17^{b}	4.31^{a}	44.27^{a}
		12.5	22.11^{a}	2.70^{a}	3.83^{b}	19.07^{b}	2.70^{a}	39.88^{b}
		C. V. (%)	17.15	13.33	12.49	15.19	12.38	17.43
	10th	Control	17.50^{a}	3.40^{a}	7.00^{a}	31.50^{a}	4.10^{a}	36.50^{a}
		6.3	16.70^{a}	1.40^{b}	5.00^{a}	27.40^{a}	1.80^{b}	37.50^{a}
		12.5	13.00^{a}	1.60^{b}	3.85^{b}	29.78^{a}	0.80^{b}	30.95^{a}
		C. V. (%)	21.06	15.70	13.13	23.56	13.73	23.34

Means followed by different letters in the columns differ statistically by Tukey's test at 5 % probability. C.V.= coefficient of variation. PL: plasmocytes; PR; prohemocytes; OE: oenocytes; SP: spherulocytes; AD: adipohemocytes; GR: granulocytes. Source: Authors.

3.2 Pupae morphological changes induced by *M. rileyi* or feeding with glyphosate

Caterpillars treated with novaluron presented significant digestive tract leakage and eczema problems (Figure 3), which caused the death of insects after the fifth day of evaluation, preventing them from reaching the pupal phase. For that reason, morphological changes were measured only in the glyphosate and *M. rileyi* treatments (Table 3).

The width and length of pupae treated with glyphosate showed no statistical difference in relation to the control nor between the doses. *M. rileyi* significantly affected pupal width, length, and weight when compared to the control, with the highest dose being most entomotoxic (no survival). When comparing treatments, all parameters were higher under glyphosate exposition (Table 3). In addition, malformations were observed in pupae and adults in the glyphosate-containing diet assays (Figure 4).

Figure 3. *Anticarsia gemmatalis* caterpillars killed by the action of the insecticide novaluron. a) Insecticide action on the intestinal epithelium. b) Insecticide action on ecdysis.

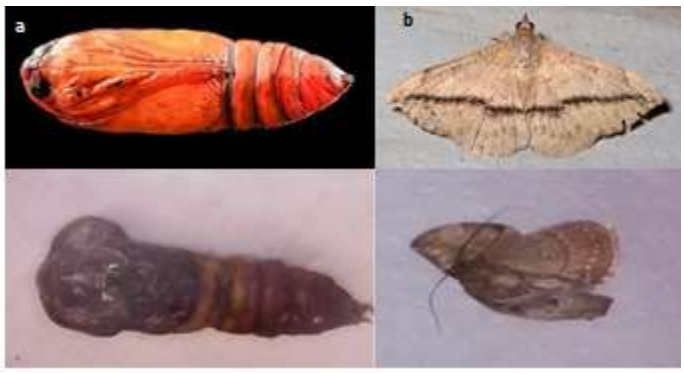
Source: Authors (2021).

Table 3. Morphological changes on *Anticarsia gemmatalis* pupae after exposition to the *Metarhizium rileyi* or fed with diet containing glyphosate.

Treatment	Dose	Width (mm)	Length (mm)	Weight (g)
M. rileyi (conidia/mL)	Control	5.09[a]	15.58[a]	0.21[a]
	10^7	1.26[b]	3.89[b]	0.06[b]
	10^9	NE	NE	NE
Glyphosate (µg/100mL)	Control	5.09[a]	15.58[a]	0.21[ab]
	6.25	4.69[a]	15.55[a]	0.22[a]
	12.5	4.65[a]	14.15[a]	0.18[b]
C.V. (%)		20.62	26.43	4.32

Different letters indicate statistically significant differences with respect to treatment (upper) and doses (lowercase), by Tukey's test at 5% probability. C.V.= coefficient of variation. N.E.: not evaluated. Source: Authors.

Figure 4. (a) Pupa of *Anticarsia gemmatalis* normal and pupa with malformation; (b) *Anticarsia gemmatalis* adult normal and adult with malformation caused by the action of glyphosate.



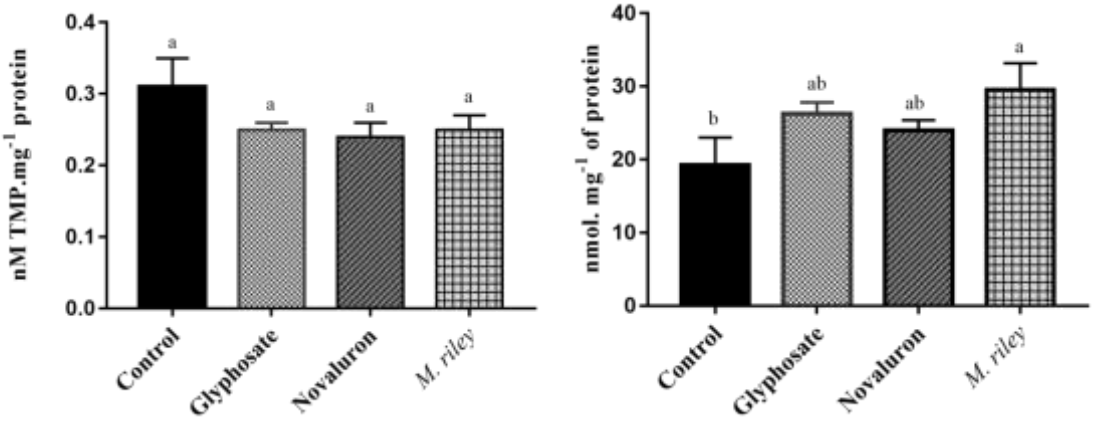
Source: Authors (2021).

The assessment of the sensitivity of the fungus to agrochemicals found that both glyphosate and novaluron interfere with mycelial growth and entomopathogen sporulation, making their simultaneous use incompatible. Thus, further studies related to the application interval between the two agents are suggested so that they can be used without interfering with the respective mechanisms of action (data not shown).

3.3 Caterpillar redox metabolism changes under novaluron, glyphosate, and *M. rileyi* treatments

To help clarify the cytotoxic mechanisms of novaluron, glyphosate, and *M. rileyi*, oxidative stress parameters were evaluated by means of oxidative damage to lipids (TBARS) and nitric oxide (•NO) levels (Figure 5). Significant statistical differences were not observed on lipid peroxidation in all treatments (Figure 5 A). However, levels of •NO were significantly augmented under *M. rileyi* treatment (53%). Although without significant difference, novaluron and glyphosate increased •NO levels, by 25 and 36%, respectively (Figure 5 B).

Figure 5. Levels of oxidative damage to lipids (TBARS) (A) and nitric oxide (• NO) (B) in *Anticarsia gemmatalis* when infected with the entomopathogenic fungus *M. rileyi* or when fed with novaluron or glyphosate.



Source: Authors (2021).

4. Discussion

The present study analyzed, for the first time, the immune system, biological parameters, and oxidative stress levels of *A. gemmatalis* when infected by the entomopathogenic fungus *M. rileyi* and when fed with novaluron and glyphosate.

The major changes in insect immune system occurred under the novaluron treatment, which may have caused the death of the insects by the fifth day of treatment. In the bioassays with the entomopathogenic fungus *M. rileyi*, the changes in the immune system occurred later. Monitoring immune response is important, because a marked decrease in the total number of circulating hemocytes may indicate infiltration of hemocytes in affected tissues, low replacement of cells by hematopoietic tissues, or inefficiency of the immune system and it is associated with apoptosis and consequent mortality of the caterpillars (Costa & Martins, 2009). The reduction in THC found in the present study was also observed in *Agrotis ipsilon* caterpillars treated with the insecticide Dimilin (El-Aziz & Awad, 2010), in *Spodoptera litura* treated with Neem gold (Sharma et al., 2003), and in *Mythimna separata* exposed to sublethal doses of hexaflumuron (Huang et al., 2016), which is a growth-regulating insecticide similar to the novaluron.

No reported studies have quantified hemocytes with *M. rileyi* infecting Lepidoptera. Interestingly, entomopathogenic fungi have a variety of mechanisms that can neutralize the host defenses, such as the production of secondary metabolites able to suppress the insect immune system (Dubovskiy et al., 2013). Fiorotti et al. (2019) demonstrated that the entomopathogen *Metarhizium robertsii* negatively affected *Rhipicephalus microplus* tick hemocytes, probably causing cell death. De Paulo et al. (2018), in the hemocyte quantifications performed after inoculation in *M. robertsii* and *M. anisopliae*, found notable changes in the number of hemocytes in *R. microplus*, with reduction of approximately 80% in females.

In our DHC analysis, the plasmocytes, prohemocytes, oenocytes, spherulocytes, adipohemocytes, and granulocytes presented the same morphological pattern found by other authors for the order Lepidoptera (Gupta, 1979, 1991; Falleiros et al., 2003; Nardi, 2004; Negreiro & Andrade, 2004). Under novaluron exposition, DHC analysis was similar to the data obtained by El-Aziz & Awad (2010), who verified that prohemocytes and plasmocytes decreased significantly and granulocytes increased in *A. ipsilon* treated with the insecticide dimilin. Similar resultas were described by s Sharma et al. (2008) in *S. litura* treated with the essential oil of *Acorus calamus*.

In the treatment with *M. riley* at the lowest dose, a process of recognition and attempted defense occurred through the increase of plasmocytes and granulocytes, which have encapsulation function in most Lepidoptera. The prohemocytes, which are the stem cells, diminished, evidencing differentiated in defense cells. The number of adipohemocytes also decreased. In the present study, the significant reduction in the number of spherulocytes induced by novaluron demonstrates that despite the defense attempt, the immune system declines, except for the granulocytes that are involved in encapsulating the pathogen. Spherulocytes are cells responsible for prophenoloxidase and melanization in Lepidoptera, which are related to the tissue renewal, transport of substances (like hormones), and production of some proteins (Negreiro & Andrade, 2004).

The results obtained with the *M. rileyi* corroborate with those reported by El-Aziz & Awad (2010), who verified in *A. ipsilon* infected with *B. thuringiensis* that plasmocytes, prohemocytes, and granulocytes increased at the beginning of the infection, but then decreased significantly. The reduction in spherulocytes may indicate that despite the mobilization of the immune system through constant production and a slight increase in prohemocytes (steam cell), the death of the insect may occur in the next few days. Adipohemocytes, cells that contain dense granules with energy-providing lipids, were also reduced, justifying the imminent death of the insect (Butt & Shields, 1996).

With the glyphosate, the number of plasmocytes, spherulocytes, and granulocytes had small oscillations, which may indicate that, despite the immune system being reached, the insect could survive. Hemocytes are extremely relevant for studies about the host-pathogen interactions because knowledge of the immunological responses in the hemolymph of insects can provide help to design of new forms of biological control (Silva, 2002), since these responses occur when the pathogen successfully overcomes the host's defense system (Falleiros et al., 2003). After the pathogen overcomes the barriers and reaches the insect's hemocele, it triggers the hemocyte defense, which is the immune response (Bulet et al., 1999) and the results described here could provide better understanding about these interactions.

Although glyphosate did not cause morphometric changes, it produced malformations in pupae and adults. In addition, glyphosate was not able to induce oxidative damage to lipids in *A. gemmatalis*. This can be attributed to the low dose used (12.5 µl/100 ml), which corresponds to 25 % of the recommended field dose. De Aguiar et al. (2016) investigated the effects of a glyphosate-based herbicide (1–5 mg/l) in *Drosophila melanogaster* and found no significant changes in the lipid peroxidation levels in both exposure periods (24 and 96 h).

We demonstrated that the increased levels of •NO in the treatment of *M. rileyi* could have caused the death of the insects, through the oxidative stress mechanism, corroborating with described by Branco et al. (2014, 2016). •NO is potentially toxic, particularly involved with oxidative stress (Moncada & Higgs, 2006). Moreover, it is an important cellular mediator playing a role in several biological essential processes, such as immune response against pathogens and other stress conditions (Faraldo et al., 2005; Foley & O'Farrell, 2003). The augment in •NO levels found in this study can be understood as an attempted defense by the metabolism of *A. gemmatalis*.

5. Conclusion

The results found here demonstrate, for the first time, the activation of the insect defense mechanism due to the entomopathogenic fungus *M. rileyi* and the agrochemicals novaluron and glyphosate. Furthermore, it is extremely important to

deep in the understanding of the mechanisms of action of fungi used in biological control and their effects on the physiology of their hosts for the development of new commercial products as well as their compatibility with existing products. The insecticide novaluron was able to significantly affect the distribution of cells of defense in *A. gemmatalis*, with a similar effect to the lowest dose of *M. rileyi*. Malformations in pupa and adult *A. gemmatalis* were more prominent after glyphosate administration. In treatments with *M. rileyi*, we can conclude that oxidative stress caused of the death of these insects. Taken together, the results may contribute to the design of new strategies involving biological control in late immunological modulation and redox modulation on insect physiology.

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