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Responses of Freshwater Planarian *Girardia tigrina* to Fipronil-Based Insecticide: Survival, Behavioral and Physiological Endpoints

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Abstract: Fipronil is a pyrazole insecticide used to control undesirable insect populations. Due to its large-scale application, there is the potential for surface waters' contamination, with toxic action for non-target organisms, and consequent impacts on aquatic ecosystems. Planarians are potential non-target aquatic invertebrates to these insecticides. They are widespread in tropical freshwaters and have been proposed as good candidates to assess the toxic effects of freshwater systems contaminated by insecticides. Thus, the present study aims to evaluate the sublethal concentrations of a fipronil-based insecticide that may affect the planarian physiology. After chronic exposure to Regent 800 WG®, a significant decrease in locomotor velocity (LOEC—6.25 mg·L^{-1}), regeneration of the auricles and photoreceptors (LOEC—3.13 mg·L^{-1}), and reproduction (fecundity—LOEC 12.5 mg·L^{-1}) were observed. The results of our study demonstrate that long-term exposure to a pyrazole insecticide can compromise non-target aquatic invertebrates while reinforcing the need for a better investigation of complementary parameters (such as behavior, regeneration, and reproduction) for a more accurate risk assessment of commercial pesticide toxicity in freshwater systems.

Keywords: reproduction; regeneration; freshwater ecosystems; turbellaria; chronic exposures

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Citation: dos Reis, E.B.; Farnese, F.S.; Oliveira, M.S.; Rodrigues, A.C.M.; Dornelas, A.S.P.; Sarmento, R.A.; de Souza, J.C.P.; Resende, E.C.; Saraiva, A.S. Responses of Freshwater Planarian *Girardia tigrina* to Fipronil-Based Insecticide: Survival, Behavioral and Physiological Endpoints. *Diversity* **2022**, *14*, 698. https://doi.org/10.3390/d14090698

Academic Editor: Aleš Gregorc

Received: 11 July 2022
Accepted: 19 August 2022
Published: 23 August 2022

1. Introduction

The industrialization and modernization of agriculture have allowed for increased productivity and reduced losses in the production process, while they may result in a system dependent on the use of pesticides [1]. These compounds, especially when misused and without proper technical support, can negatively impact the natural ecosystems and their biodiversity (water, soil, air, and human health) [2–5].

Numerous active ingredients and metabolites of pesticides have been reported to reach or have been found in freshwater ecosystems, and the toxicity of these compounds can affect the aquatic ecosystem's health. For example, the insecticide fipronil belongs to the chemical group of phenylpyrazoles and is used in the agricultural sector to control insect plagues. The fipronil-based insecticides used in agriculture and livestock can reach freshwater ecosystems, and concentrations up to 465 µg·L^{-1} have been reported for surface waters [6]. In agriculture, different doses of fipronil are applied to different crops, such as cotton (48 g for product compound·ha^{-1}), potato (175 g product compound·ha^{-1}), sugarcane (408 g product compound·ha^{-1}), corn (100 g product compound·ha^{-1}), and soybean (40 g product compound·ha^{-1}) [7]. These doses are considerably high and can be leached into surface water.

Scientific research has been developed to quantify pesticides with active ingredients based on fipronil in aquatic ecosystems. Some studies report that the high toxicity of fipronil-based insecticides contaminates aquatic systems and promotes lethal and sublethal effects in non-target aquatic organisms, such as fish, reptiles, and arthropods [8,9]. These facts result in an imbalance in these ecosystems [10–12].

Fipronil is a neurotoxic insecticide that acts by causing paralysis and neural excitation, leading to the death of insects. The compound comes into contact with the body through the skin and/or by ingestion. When bound to the chlorine channel, this insecticide prevents its activation by gamma-aminobutyric acid (GABA) (a substance that controls the entry of chloride ions through the nerve cell membrane). When there is no synaptic inhibition, hyperexcitation of the central nervous system is provoked, causing the death of the organism [13]. The toxicity of fipronil varies from species to species. Several fish species are quite sensitive to the effects of fipronil; for example, the gills of Caspian whitefish are affected by this compound [14]. Furthermore, changes in physiology and behavior were analyzed in *Daphnia magna* by exposure to fipronil [15]. Moreover, fipronil accumulates in Nile tilapia tissue, leading to toxicity for cells and genes, and-mortality, which characterizes the compound as very toxic for this species [16].

Planarians belong to the tubelar group and reproduce both asexually and sexually (in some species, reproduction occurs in both ways). Planarians found in lakes and streams have a high capacity for regeneration. Tubelars have unique characteristics, such as a nervous system similar to the vertebrate brain, which makes them essential organisms for several areas of research, due to the varied parameters that can be evaluated in response to chemical stress, and the high sensitivity to toxic compounds [17].

Planarians occur naturally in several freshwater environments (lakes, streams, and rivers) that are frequently located close to agricultural areas, and can be directly exposed to insecticides, as well as exhibit sensitivity to various toxic agents [18]. These flatworms have important roles in freshwater food webs by being predatory invertebrates, as well as food for other organisms (prey). In addition, planarians are organisms that are easy to maintain in the laboratory, which reduces the cost and facilitates the collection of ecotoxicological data [19]. Due to these factors, planarians become suitable candidates for test organisms in ecotoxicology [20,21]. Studies indicate that organisms exposed to contaminants have shown changes in the reproductive system, as well as in fecundity [22]. In the field of ecotoxicological freshwater, planarians of the species *Girardia tigrina* (Paludicola, Dugesiidae) have been recently used as bioindicator organisms of environmental contamination. These aquatic invertebrates allow for the measurement of behavioral (locomotion and feeding), regenerative (head, auricles, and photoreceptors regeneration), and reproductive (fecundity) parameters to assess the toxicity of pesticides [23–26]. Thus, this study aims to evaluate the potentially sub-lethal effects of the commercial Regent 800 WG (fipronil-based insecticide) using sensitive endpoints at the behavioral, regenerative, and reproductive levels on the non-target freshwater planarian *G. tigrina*.

2. Materials and Methods

2.1. *Girardia tigrina*

Planarians (*G. tigrina*) were obtained from established cultures at the Laboratory of Applied Studies in Plant Physiology—in partnership of CAE (Conservation of Agroecosystems and Ecotoxicology) group, in the Instituto Federal de Educação, Ciência e Tecnologia Goiano—Campus Rio Verde. Planarians were grown in American Standard Test and Materials (ASTM) aqueous media (a medium composed of solutions of sodium bicarbonate, magnesium sulfate, potassium chloride, calcium sulfate, and distilled water), under a controlled temperature of 22 ± 1 °C, in the dark [27].

Once a week, the planarians were fed, *ad libitum*, bovine liver, and the medium was renewed two hours after feeding. One week before the experiments, the organisms were deprived of food to avoid contamination by digesting food and guaranteeing homogeneity in the physiological state between organisms [28].

For the assays, the planarians were measured in a checkered mesh and animals with no signs of injuries were selected. For the chronic toxicity test (locomotion and regeneration), organisms with 1.0 ± 0.2 cm were selected. For the chronic test (reproduction), the planarians were 1.5 ± 0.1 cm long.

2.2. Preparation of the Fipronil-Based Insecticide

A stock solution of insecticide based on fipronil was prepared using 6250 mg·L^{-1} of the Regent 800 WG® granulated of the equivalent compound of fipronil/L (with a final concentration of 5000 mg·L^{-1}), in distilled water. This proportion was performed through the proportion calculation. The formulated compound was chosen due to environmental relevance, since in agricultural areas it is more common to use the commercial compound than the active ingredient in a pure state.

The stock solution was kept in the absence of light at 4 °C to avoid the degradation of the active ingredient. The experimental solutions were prepared by diluting stock solution in ASTM medium. This solution was prepared based on a proportionality rule, so that the solution had 100% of the active ingredients of fipronil.

2.3. Planarians Exposure for Locomotion and Regeneration Evaluation

Assessment of locomotion and regeneration was performed by using planarians, which were previously exposed for eight days at different nominal concentrations of fipronil insecticide: 1.56, 3.13, 6.25, 12.5, 25 mg (active ingredient)·L^{-1}, and the control experiment (ASTM). The tests were carried out at a temperature of 22 ± 1 °C, in a static system and in the absence of light. The exposure was carried out with 30 organisms per treatment, divided into three replicates (with 10 planarians per replicate), in glass beakers containing 100 mL of experimental solution. After the fourth day of exposure, the experimental solutions were renewed. At the end of the exposure period, the effects on locomotion and regeneration were evaluated (adapted from [28–30]).

2.3.1. Planarian Locomotor Velocity—*p*LMV

The locomotor velocity of the planarian (*p*LMV) was evaluated using a round shape (25 cm in diameter) covered with millimeter paper (lines spaced at 0.5 cm) and glued with transparent sticky paper. After 8 days of exposure to *p*LMV, the planarians were evaluated at all concentrations. The bottom of the mold was covered with ASTM medium, and the planarians' behavior was evaluated individually. After thirty seconds of adaptation, the locomotor behavior of the planarians was monitored by counting the millimeters traveled for two minutes. Each time the planarians crossed a line, 5 mm was counted (adapted from [28–30]).

2.3.2. Regeneration

In the regeneration step, fifteen planarians per concentration were decapitated by a single precise cut behind the auricle, using a previously sterilized scalpel blade. After decapitation, the planarians were individually transferred to a polyethylene terephthalate (PET) flask containing 20 mL of ASTM medium. Then, the regeneration was analyzed by monitoring the number of hours (every 24 h) until the formation of new photoreceptors and auricles, as well as the complete regeneration of the head by using a magnifying glass Magnifier Lamp (adapted from [28–30]).

2.3.3. Reproduction

At the beginning of the reproductive phase (1.5 ± 0.1 cm in total length), adult planarians were exposed to different concentrations of fipronil (Regent 800 WG) for 3 weeks to assess fecundity, and for 4 weeks to assess hatching cocoons (fertility). They were exposed at five different concentrations of fipronil (1.56, 3.13, 6.25, 12.5, and 25 mg·L^{-1}), and control treatment (only ASTM medium), in triplicate, each replicate containing 10 organisms. These organisms were exposed to 100 mL of experimental solution in PET flasks. Weekly, after feeding the organisms with bovine liver (*ad libitum*), the solutions of each concentration were replaced by new solutions. The experiment was carried out at a temperature of 22 ± 1 °C, in the absence of light, and the animals were observed daily. Each deposited cocoon was placed in a PET container with 20 mL of experimental solution. Fertility was assessed by the number of cocoons produced per day divided by the number of planarians exposed. The fecundity rate was determined by the number of offspring (planarians born from the cocoons), divided by the number of cocoons deposited (adapted from [28,31]).

2.4. Statistical Analysis

The sublethal toxicity data were evaluated by analysis of variance (ANOVA), and Dunnett's post hoc test was applied to assess whether there were significant differences between treatments. In order to verify whether the data were in accordance with ANOVA's assumptions, the data were analyzed for homogeneity of variances and normality, using the Bartlett and Kolmogorov–Smirnov tests, respectively. Data obtained from the regeneration tests did not follow the assumptions of the ANOVA analysis; therefore, it was necessary to use nonparametric statistics using the Kruskal–Wallis test (Dunn's post hoc test). Statistical analysis was performed using GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Planarian Locomotor Velocity—pLMV after Exposure to the Regent 800 WG®

The planarians' locomotor velocity (*p*LMV) decreased significantly after exposure to Regent 800 WG®, when compared to the control treatment ($F_{(5,84)} = 11.1$; $p < 0.0001$, R = 0.3979), presenting a NOEC (no observed effect concentration) of 3.13 mg·L^{-1}, and a LOEC (lowest observed effect concentration) of 6.25 mg·L^{-1} (Figure 1).

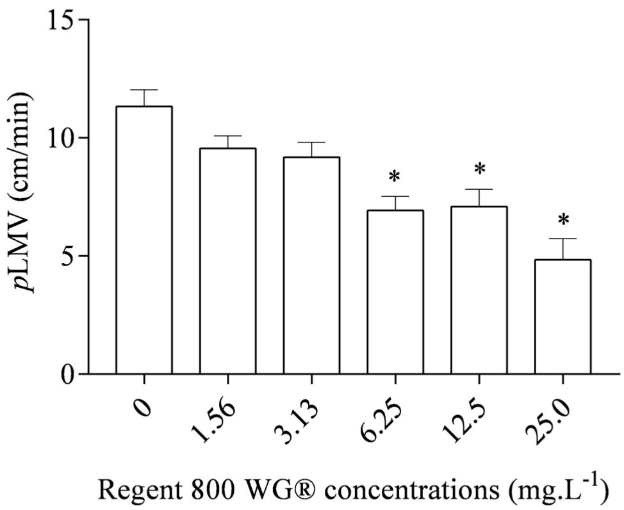

Figure 1. The *p*LMV of the *G. tigrina*, after eight days of exposure to sublethal concentrations of the Regent 800 WG®. Concentrations refer to active ingredient of fipronil. Data are presented as mean ± standard error, *n* = 15. * Significant differences are observed in comparison with the control treatment (Dunnett's post hoc test, $p < 0.05$).

3.2. Regeneration of Planarians after Exposure to Regent 800 WG®

As a result of the increase in Regent 800 WG® concentrations, the exposed planarians suffered a significant delay in the auricles regeneration ($F_{(5,84)} = 87.89$; $p < 0.0001$; R = 0.8394; Figure 2b) compared to the control treatment. Similarly, the regeneration of photoreceptors was significantly delayed with the increasing insecticide concentrations (H = 77.5; $p < 0.0001$; Figure 2a). NOEC was set at 1.56 mg·L^{-1}, and LOEC was set at 3.13 mg·L^{-1} for the regeneration of photoreceptors and auricles.

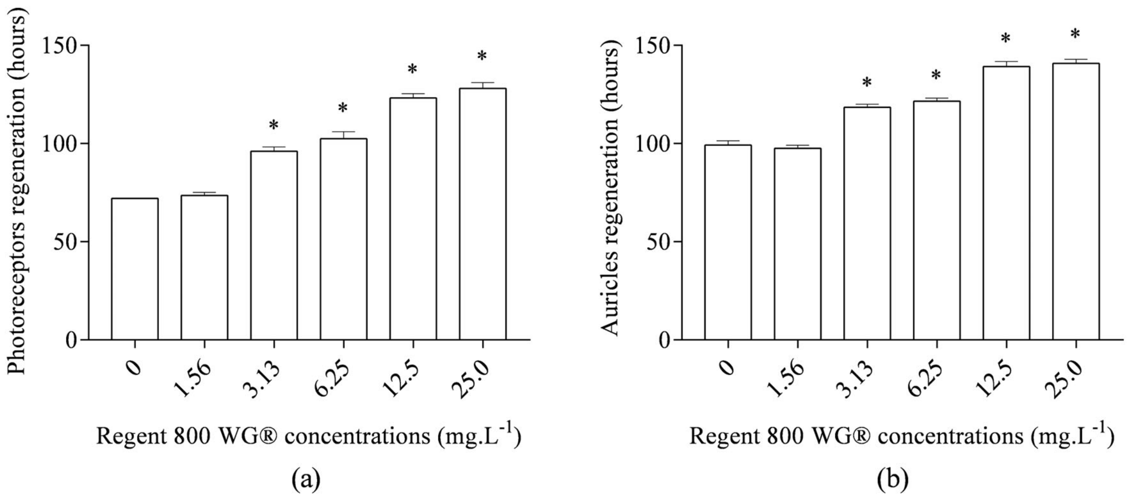

Figure 2. Effects of sublethal concentrations of Regent 800 WG® insecticide on the regeneration of the *G. tigrina*. (**a**) Photoreceptor regeneration. (**b**) Auricles regeneration. Concentrations refer to active ingredient of fipronil. The exposure time was sixteen days. Data are presented as mean ± standard error, $n = 15$. * Significant differences are observed in comparison with the control treatment (Dunn's post hoc test).

3.3. Reproduction of Planarians Exposed to the Regent 800 WG®

The fecundity rate of *G. tigrina* decreased significantly with the increase of the Regent 800 WG® concentrations ($F_{(5,10)} = 4.875$; $p < 0.05$; R = 0.7187; Figure 3a). The NOEC for fecundity rate was established at the concentration of 6.25 mg L^{-1}, and the LOEC at the concentration of 12.5 mg·L^{-1}. However, exposure to the tested concentrations of the pesticide did not significantly affect the fertility rate of *G. tigrina* ($F_{(5,9)} = 1.886$; $p > 0.05$; R = 0.213; Figure 3b).

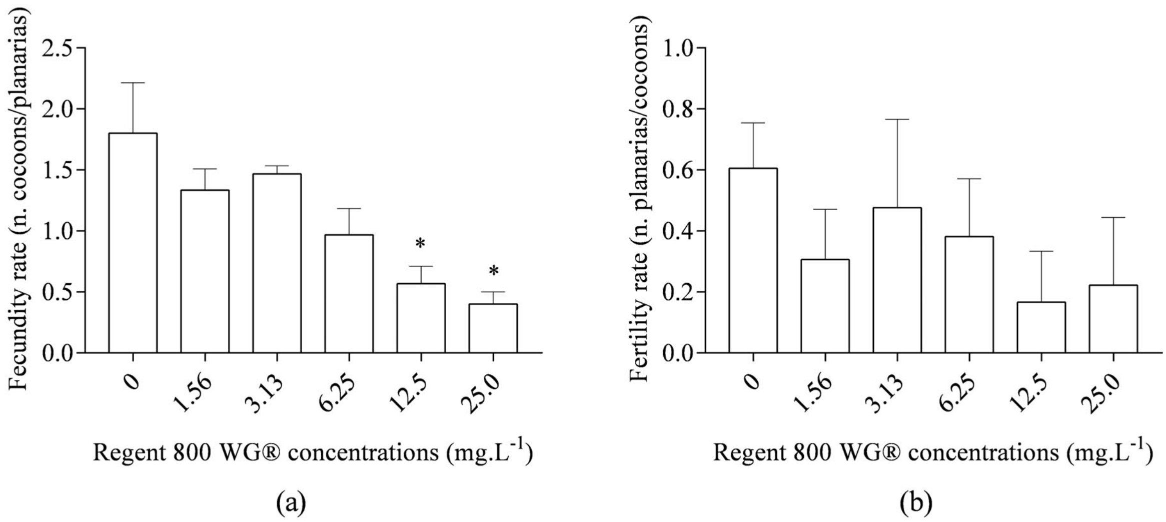

Figure 3. Effects of sublethal concentrations of Regent 800 WG® on the reproduction of the *G. tigrina*. (**a**) Fecundity rate. (**b**) Fertility rate. Concentrations refer to active ingredient of fipronil. Data are presented as mean ± standard error, $n = 30$. * Significant differences are observed in comparison with the control treatment (Dunn's post hoc test).

4. Discussion

Although studies on the impact of fipronil on non-target organisms have been more notorious in recent years, there are still few studies addressing the sublethal effects of fipronil-based insecticides in aquatic invertebrates [32–37]. It is noteworthy, however, that concerning aquatic invertebrates, there is a pronounced lack of data on the effects of fipronil both in the short and in the long term, which highlights the novelty and importance of this study.

Regarding the chronic effects on non-target organisms, fipronil altered the reproduction of the collembola species *Folsomia candida* at concentrations lower than those recommended for use in agriculture [38]. It also was highly toxic to bees, impairing the learning behavior of the species *Apis mellifera* [39]. In addition, aquatic organisms such as amphibians, cladocerans, and oligochaetes showed harmful effects related to exposure to fipronil [40].

Understanding and gaining deep knowledge about the changes due to chronic toxicity at the organism level is important for understanding the long-term toxicity of xenobiotics to populations and the structure and function of the natural communities. In this study, locomotor behavior was evaluated using a traditional method, where the user made observations by the naked eye and not by a video-tracking system. Despite the known limitations of this method, evaluating only straightforward movements of planarians is a very simple, rapid, and cheap method to apply with a good sensitivity to detect the sublethal effects of the several toxicants reported [41]. The locomotor behavior of the planarians was affected with a LOEC of 6.25 mg·L^{-1}. This negative effect possibly occurred due to fipronil affecting the nervous system of the planarians. In fact, the locomotor behavior of the planarians is related to the proper functioning of the nervous system controlling muscle contraction [42]. Furthermore, the feeding activity of the planarians is directly related to their locomotor capacity. Planarians have an intermediate position in freshwater food chains; thus, changes on their predator and escape behavior may compromise the individual's survival, ultimately generating both top-down and bottom-up imbalances in these food chains [19,23].

The regeneration of *G. tigrina* was the most sensitive parameter after exposure to fipronil compared with the other parameters evaluated. There was a significant delay in the regeneration of photoreceptors and auricles from the concentration of 3.13 mg·L^{-1} of fipronil. These results reinforce the potential toxic action of fipronil towards the nervous system of planarians. The regeneration of planarians depends on muscle contraction and the extension of the epidermis in the wound, a process that leads to the closure of the lesion. As a result of these changes, the cells identified as blastema are organized under the epidermis and the replacement of missing segments is initiated [43,44]. As a consequence of the delay in the regeneration of photoreceptors, planarians can suffer changes in the perception of the intensity and direction of light. Similarly, the delay in the regeneration of auricles can limit the perception of the chemical detection from the surrounding environment, including chemical clues to detect preys as well as predators [18].

There was also a reduction in the fertility rate of planarians, presenting a LOEC of 12.5 mg·L^{-1}. In a study carried out with *Danio rerio*, it was found that when exposed to concentrations of 2.5 mg·L^{-1}, their incubation capacity (in the embryo stage) is affected [45]. This toxic action of fipronil through neural damage, hepatotoxic, nephrotoxic, cytotoxic, and reproductive effects in both vertebrates and invertebrates were observed [46].

Surface waters can be contaminated by pesticides as a result of leaching and surface runoff, due to the overdose and misuse of these products. These events facilitate insecticide spread to the aquatic environment, and can cause impacts on aquatic ecosystems, and consequently affect non-target organisms (such as the planarians) [47]. In sum, our study highlights that prolonged exposures to fipronil-based insecticides might impair the behavior, regeneration capability, and reproduction of *G. tigrina* in similar concentration ranges, despite regeneration being the most sensitive parameter. Furthermore, these parameters are the visible outcome of a series of biochemical and physiological changes in response to exposure to stressors, therefore indicating changes at higher levels of biological organization [48]. In the present study, we use the commercial formula containing around 80% fipronil and 20% other components. The presence of this 20% of a mixture of compounds can cause some discussion about their possible toxic action on planarians. However, evaluating the toxicity of the commercial formula, we present more relevant data for a realistic risk assessment of fipronil-based insecticides to non-target freshwater animals nearby agricultural areas. Although fipronil has low to moderate water solubility, due to its widespread use, the insecticide is currently present in soils and surface and groundwater, and direct or indirect effects on aquatic organisms can be expected [49]. Thus, we speculate the potential deleterious effects of the commercial compound Regent 800 WG® on tropical freshwater planarians, considering that in an aquatic environment, without the presence of light (places inhabited by planarians), the half-life of fipronil may be relatively long—with a half-life ranging between 36 h and 7.3 months depending on substrate and conditions [50]. In addition, fipronil has slight mobility in soil, a bioconcentration factor within the limit of concern, a leaching rating—GUS Index of 2.06 (considered transitional range), and high toxicity to sediment-dwelling aquatic organisms [51]. These results are thus of concern regarding natural population dynamics, since planarians' development and reproduction success might be decreased, leading to changes in freshwater communities.

5. Conclusions

Fipronil-based insecticide has been shown to cause chronic effects in planarians (*G. tigrina*), such as delay in locomotion (from concentration 6.25 $mg \cdot L^{-1}$), regeneration (from concentration 3.13 $mg \cdot L^{-1}$), and reproduction (fecundity, from concentration 12.5 $mg \cdot L^{-1}$). These results will certainly contribute, with important ecotoxicological data, to the research about the chronic effects of fipronil on tropical freshwater invertebrates, contributing to a more sensitive approach to the analysis of the ecological risk in these climates represented by fipronil or other insecticides of the pyrazole group. In addition, this study reinforces the importance of sublethal tests and evaluations of individual parameters (locomotion, regeneration, and sexual reproduction of planarians), which tend to be much more sensitive than survival bioassays.

Author Contributions: E.B.d.R. conducted experiments; E.B.d.R. and A.S.S. analyzed data and wrote the manuscript (writing—original draft, review, and editing); F.S.F., M.S.O., A.C.M.R., A.S.P.D., R.A.S., J.C.P.d.S. and E.C.R. analyzed data, reviewed, and edited the manuscript; A.S.S. and F.S.F. conceived (funding acquisition) and designed research. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Programa de Apoio à Pós-Graduação—PROAP/CAPES IF Goiano—Programa de Mestrado em Agroquímica, 2020.

Institutional Review Board Statement: Ethical review and approval were waived for this study due to REASON (The existing legislation on the welfare of experimental animals is not applicable for organisms used in this study. In addition, this study did not involve the collection of endangered or protected animal species).

Data Availability Statement: Not applicable.

Acknowledgments: We would like to thank the Programa de Mestrado em Agroquímica of the Instituto Federal de Educação, Ciência e Tecnologia Goiano—Campus Rio Verde and the Instituto Federal de Educação, Ciência e Tecnologia Goiano—Campus Campos Belos and Campus Iporá for support and partnership. We thank the Programa de Apoio à Pós-Graduação—PROAP/CAPES IF Goiano— Programa de Mestrado em Agroquímica 2020. Multi-User Analytical Center (CeMA) of the Programa de Mestrado em Agroquímica (PPGAq) and M.S.O for the scholarship PNPD/CAPES No. 88887.342460/2019-00. A.C.M.R acknowledge the financial support to CESAM by the FCT/MCTES (UIDP/50017/2020 + UIDB/50017/2020 + LA/P/0094/2020), through national funds. A.S.P.D acknowledge Capes, for the PNPD Grant, No. 88887.666008/2022-00. Finally, we would like to thank the Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação—Instituto Federal de Educação, Ciência e Tecnologia Goiano, for the financial assistance in processing the article.

Conflicts of Interest: The authors declare no conflict of interest.

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