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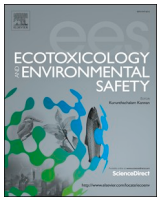

Impact of temperature on the toxicity of Kraft 36 EC® (a.s. abamectin) and Score 250 EC® (a.s. difenoconazole) to soil organisms under realistic environmental exposure scenarios

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ABSTRACT

Pesticides can affect all receiving compartments, especially soils, and their fate and effects may be enhanced by temperature, increasing their risk to ecological functions of soils. In Brazil, the most widely used pesticides are the insecticide Kraft 36 EC® (a.s. abamectin) and the fungicide Score 250 EC® (a.s. difenoconazole), which are commonly used in strawberry, often simultaneously as a mixture. The aim of this study was to evaluate the toxicity of realistic environmental applications, single and in mixtures, for both pesticides to the springtail *Folsomia candida* and the plant species *Allium cepa* (onion) and *Lycopersicum esculentum* (tomato). Mesocosms filled with Brazilian natural soil (lattosolo) were dosed with water (control), Kraft (10.8 g a.s/ha), Score (20 g.a.s/ha) and Kraft + Score (10.8 + 20 g a.s./ha). The applications were repeated every 7 days, during 18 days of experiment, and simulating rainfall twice a week. Collembola reproduction tests were conducted with soils from the first (day 1) and last day (day 18) of experiment for each treatment. Plant toxicity tests were carried out in the experimental units. The experiments were run at 23 °C and 33 °C. Kraft, alone and in the binary mixture, showed high toxicity to the springtails in soils from both days 1 and 18, especially at 23 °C where it caused 100% mortality. Score however, was not toxic to the springtails. Plant growth was reduced by Score, but responses varied depending on temperature. This study indicates a high environmental risk of the insecticide Kraft, particularly at lower temperatures (23 °C), and an influence of temperature on pesticide fate and effects.

1. Introduction

It has generally been acknowledged that temperature may impact the toxicity of contaminants as well as on their distribution over different environmental compartments (Bell et al., 2007; Van den Brink et al., 2018; Noyes et al., 2009; Schiedek et al., 2007). Pesticides may pollute the environment through different routes (Vryzas, 2018) and have been detected in all environmental compartments (Estévez et al., 2012; Lapworth et al., 2012). Most studies evaluating pesticides have focused on surface water (Chelinho et al., 2012; Kuster et al., 2008; Murray et al., 2010; Niemeyer et al., 2017), even though soils and sediments have been indicated as the largest deposits of these compounds (Vryzas, 2018), which may lead to significant risks to the ecological functions of soils, plant growth and even human health (Sun et al., 2018).

Brazil is currently the world's largest consumer of pesticides, accounting for approximately 20% of the total global use (Albuquerque et al., 2016). Brazil is also the third largest fruit producer in the world after China and India (FAO, 2017). For example, strawberry production in Brazil heavily depends on the use of the insecticide/acaricide Kraft 36 EC® (a.s. abamectin) and the fungicide Score 250 EC® (a.s. difenoconazole) (Pitombeira de Figueirêdo et al., 2019), which are often used simultaneously as a mixture (Novelli et al., 2012; Sanches et al., 2017).

In recent years, studies have demonstrated a high toxicity of Score 250 EC® and especially Kraft 36 EC® to non-target organisms

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(Moreira et al., 2017; Nunes et al., 2016; Nunes and Espíndola, 2012; Pitombeira de Figueirêdo et al., 2019). For example, Oliveira et al. (2018), evaluating their toxicity to the potworm *Enchytraeus crypticus*, the collembolan *Folsomia candida*, and the mite *Hypoaspis aculeifer*, concluded that environmentally relevant concentrations of both pesticides may significantly affect the reproduction of Collembola (Oliveira et al., 2018). In addition, mixtures of the two pesticides showed synergistic effects on aquatic organisms, indicating that environmental risk evaluations based on individual compounds may not adequately protect aquatic ecosystems (Moreira et al., 2017; Sanches et al., 2017). Besides, most studies evaluating these compounds were conducted with a concentration series to determine toxicity values (e.g. EC_{50}, NOEC). It therefore is still unclear how these toxicity values relate to actual risks in the environment under realistic exposure scenarios.

The aim of the present study was to evaluate the toxicity of Kraft 36 EC® and Score 250 EC®, single and in mixture, to the collembolan *Folsomia candida* and two plant species (the onion *Allium cepa* and the tomato *Lycopersicum esculentum* Mill). A mesocosm experiment was conducted simulating a realistic pesticide application scenario and the toxicity was evaluated by conducting the experiments at 23 °C and 33 °C.

2. Materials and methods

2.1. Mesocosm experiment

Mesocosms (n = 12) were constructed in fiberglass jars of cylindrical shape (0.52 m in diameter, 0.67 m in height), filled with 100 kg natural soil (lattosolo, 11.06% organic matter; cation exchange capacity (CEC) 3.52 ($cmol_c$/kg); 35% clay, 21% silt, 22% fine sand, 20% medium sand, 2% coarse sand). The natural soil was collected at the field station of Center for Water Resources and Environmental Studies (CRHEA/ 22°01′22″S, 43°57′38″W), São Carlos School of Engineering (EESC), University of São Paulo (USP), that has not received application of pesticides in the last 25 years (Chelinho et al., 2012; Nunes and Espíndola, 2012). The soil was sieved through approximately 5 mm and dried under a controlled temperature of 60 °C in an oven. After allowing the mesocosms to stabilize for 3 days, the following treatments were prepared with 3 replicates each: Kraft (K), Score (S), Kraft + Score (K + S) and control. Following the manufacturer's instructions for strawberry crop, the doses applied corresponded for Kraft 36 EC® with 10.8 g abamectin/ha and for Score 250 EC® with 20 g difenoconazole/ha. Application solutions of 1 L Milli-Q® water with the single compounds were apply the dose in each mesocosm (filled with air-dried soil obtained as described above), for mixtures, the solutions were prepared in 0.5 L Milli-Q® water, thus also counting 1 L of solution for contaminating the simulators (Table S1). Pesticides solutions were individually applied directly on soil, once a day, with the help of hand sprayers for approximately 30 min. Also following the manufacturer's instructions, the pesticide applications were repeated 7 and 14 days following the first application, with a total test duration of 18 days. Experiments were conducted at 23 °C and 33 °C in separate temperature-controlled climate rooms. These temperatures correspond with temperatures measured in the region (Ferrari, 2012; Neves et al., 2018; Soares et al., 2012). After the first pesticide application (day 0), 35 L uncontaminated water (corresponding to 225 mm of rain, average of the wettest months in the region) was sprayed on the surface of each mesocosm. Another five rain events (twice a week) were simulated by applying 6 L (on pesticide application days, 5 L lake water and 1 L pesticide solution) to each mesocosm, corresponding to 37.5 mm which is the approximate average of months with less precipitation (INMET, 2019).

To evaluate the impact of the pesticide applications, toxicity tests were conducted with the collembolan *Folsomia candida* and two plant species, one monocotyledone (*Allium cepa*, cultivar: Baia Periforme) and one dicotyledone (*Lycopersicum esculentum Mill,* cultivar: Super Marmande).

2.2. Test organisms

A culture of *F. candida* (Collembola) has been kept in the temperature-controlled laboratory (NEEA/CRHEA/EESC-USP) for many years. Cultures are kept at 20 ± 1 °C with a 12/12 h light-dark regime, in a substrate of activated charcoal and plaster of Paris with water-saturated base. Animals were fed weekly *ad libitum* with dried baker's yeast (*Saccharomyces cerevisiae*) which was moistened to 40–60% with distillated water. The plant seeds used in the experiments were purchased from specialized stores (*A. cepa,* Feltrini Sements®, Lot: 4707S2, Germination rate: 88%; *L. esculentum Mill*, Isla Sements®, Lot: 38,452-S2, Germination rate: 85%).

2.3. Collembola tests

Reproduction tests (28 d) with *F. candida* were conducted directly after (approximately 2 h) the first pesticide application and at the end of the 18-day incubations at 23 °C and 33 °C. Tests were conducted as described in the standardized ISO protocol 11,267 (ISO, 2014). As indicated in this protocol, ten juveniles (10–12 day old) were exposed in 100 mL test jars to 30 g soil of each mesocosm. The soil was collected by taking subsamples from the surface of the mesocosms (± top 5 cm), using a spoon, from three different points, to guarantee the sampling representativeness. The sampled soil was mixed in a plastic bag and used to perform the collembolan test. Five replicates were used for each treatment. All tests were conducted in a climate room at 20 °C and a 16 h/8 h light/dark cycle. During the tests, soil moisture content and food availability were checked weekly and adjusted if needed (greater than 2% of initial water content). After the 28-d exposure period, the number of surviving adults and new-born juveniles were assessed by extracting the animals through flotation with water, after which digital photographs were made and animals were counted using the Image J software.

2.4. Plants test

After removing the soil for the tests with *F. candida*, the top soil layer was structured for planting the seeds, and the toxicity tests with *A. cepa* and *L. esculentum* were carried out in the experimental units according to the standard protocol ABNT NBR ISO 11269–2 (2014), with minor modifications due to the size of the experimental unit and the duration of the experiments. In each mesocosm, 15 seeds of each plant were planted, with equal spacing between the seeds and with intercalated arrangement. The planting was carried out immediately after pesticide treatment and removal of the soil for the Collembola tests. After 18 days, the plants were harvested, after which the length (aerial part, i.e. length of the stems and the major axis) and weight (wet and dry) were measured. The plants of each species were weighed together per mesocosm due to the low weight of the individual plants. The pooled value was divided by the total number of plants weighed per replicate to enable determination of the average weight per plant.

2.5. Chemical analyses of the test compounds

Pesticide stock solution concentrations were analytically confirmed by HPLC-DAD (Agilent 1200 series), using a C18 (4.6 × 250 mm, 5 μm) column. The isocratic mobile phase utilized was acetonitrile and acidified water, 1% with 95% acetylic acid), at an injection volume of 20 μL and a flow rate of 1 mL/min. Each stock solution was measured in triplicate on the same day of preparation and application to the mesocosms. Abamectin and difenoconazole were detected and measured at 246 nm (with retention times, RT, of 3.6 min) and 220 nm (RT = 7.3 min), respectively. The recovery rates were 98.0 ± 4.6% for abamectin and 95.4 ± 8.5% for difenoconazole and detection limits were 0.05 mg/L and 0.01 mg/L, respectively.

2.6. Statistical analyses

The generated dataset was analyzed using the Generalized Linear Model (GLM) and Generalized Linear Mixed Model (GLMM) approaches, since these are models that allow to analyze data that are independent but without homogeneity in their variance, involving effects random (time factor) (Lo and Andrews, 2015; Lopes et al., 2018). For both Collembola and plant analyses, the explicative variables were treatments (Kraft, Score, Kraft + Score and Control), temperatures (23 °C and 33 °C) and their interactions. In the case of Collembola, the collection time (first and the last experimental day) was added as random effect due to possible correlation effects.

The response variables for Collembola models were: mortality, which was considered to follow a Binomial probability distribution, and the average number of new-born juveniles per surviving adult, which was considered to follow a Gamma probability distribution. Logit and log functions were adopted as link functions for these variables, respectively. For the plant models, the response variables were wet and dry weight, stem length (both plants), and the major length (only for tomatoes). For all plant variables, a Gamma probability distribution and a log-link function were considered. The suitability of fitted models was evaluated by residual diagnostics, and the significance of estimated coefficients for each factor was verified using Wald test (Binomial distribution) and *t*-test (Gamma distribution). The diagnostic of residuals indicated that all probability distributions were appropriate to represent data variability (see FigS. 1–4 in supplementary material), as well as the model structure. All statistical tests were performed considering the control treatment at a temperature of 23 °C as reference group and a significance level of 5%. The statistical analyses were performed using R software environment (R Core Team, 2018). For the GLMM approach, the "lme4" package (Bates et al., 2015) was used.

3. Results and discussion

3.1. Toxicity to F. candida

The insecticide/acaricide Kraft 36 EC® and its mixture with the fungicide Score 250 EC® were extremely toxic to the springtail *F. candida* at 23 °C. Soil collected from the mesocosms incubated at 23 °C on the first (day 1) and the last day (day 18) of the experiment caused 100% mortality and no juveniles were produced. Soil collected from the mesocosms incubated at 33 °C, however, was much less toxic with effects on springtail survival and reproduction in the single Kraft exposure decreasing at day 18 compared to day 1 and increasing in the mixture exposure (Fig. 1). Score 250 EC® showed no toxicity to the springtails for both experiments performed at 23 °C or 33 °C (Fig. 1 and Table S2).

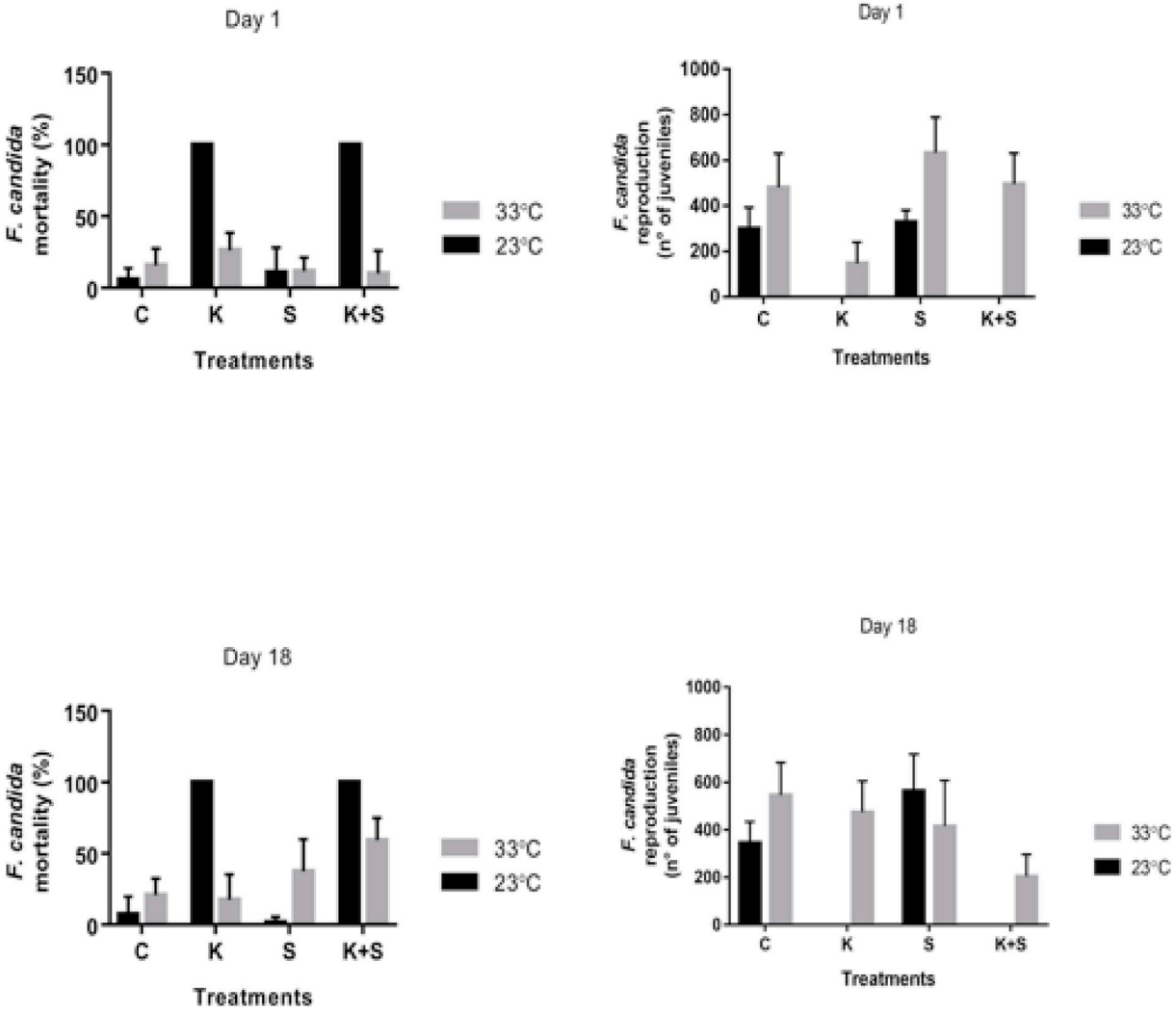

Fig. 1. Mean ± standard deviation (n = 5) mortality (%) and reproduction (number of juveniles) of *Folsomia candida* when exposed to soil taken after 1 or 18 days from mesocosms nontreated (Control, C) or treated 3 times at 7-day intervals with normal doses of abamectin in the formulation Kraft (K), difenoconazole in the formulation Score (S) or their binary mixture (K + S) and incubated at 23 °C and 33 °C.

However, even with the high toxicity (100% mortality at 23 °C on days 1 and 18) no significant difference was observed for the K and K + S estimates ($p > 0.05$). This was attributed to a statistical phenomenon called "separation" (Albert and Anderson, 1984). As the response variable has only one predicted value for these cases, the odds want to be infinite, leading to high standard error values. To demonstrate the significant effects of the interaction terms, the Likelihood ratio test (LRT) between the full model and the model without interaction was carried out. The second model presented almost twice the Akaike's Information Criterion (AIC = 1044.61) value in comparison to the full model (AIC = 540.43), indicating the significant effects of the interactions between temperature and K and K + S treatments (LRT, $\chi^2 = 510.8$, $P < 2.2e^{-16}$).

Previous studies revealed a rather large variation in the toxicity of abamectin to collembolans. Oliveira et al. (2018), for example, noted a 50% reduction in *F. candida* when exposed to 0.06 mg a.s./kg in a natural Brazilian soil (15% organic matter content; CEC 3.33 (meq/100 g); 48% clay; 12% silt; and 19, 11, and 10% of fine, medium, and coarse sand, respectively) in the laboratory. However, Diao et al. (2007) reported such a reduction at a ten-fold higher concentration in a natural (York, England) sandy-loamy soil (21% clay, 12% silt, 67% sand, 2.22% organic carbon and a pH of 7.0) (EC_{50} *F. candida* = 0.68 mg a.s./kg). In the present study, 100% mortality of *F. candida* was seen in the test conducted at 23 °C while at 33 °C no lethal effects were observed (Fig. 1). It is indeed known that soil properties have a great influence of pesticide fate and (hence) toxicity (see Daam et al., 2019 for a recent review).

At high temperatures, the faster degradation of the compound may explain for the absence of toxicity to the test organisms. The faster degradation of abamectin-based compounds at high temperatures has been reported in the literature (Awasthi et al., 2013; UE, 2011).

Jegede et al. (2017) evaluated the toxicity of three pesticides (dimethoate, chlorpyrifos and deltamethrin) to *F. candida* at 20 °C and 28 °C. They noted that the toxic effects of dimethoate and chlorpyrifos on reproduction were higher at higher temperatures, whereas toxicity of deltamethrin was greater at 20 °C. Greater toxicity of pesticides at higher temperatures has been attributed to higher uptake rates, whereas lower toxicity may be related with a faster dissipation and higher elimination rates in organisms (Daam et al., 2019; Römbke et al., 2007). Neurotoxic pesticides like abamectin, for example, have been indicated to have greater toxicity to insects at lower temperatures due to increased stability of sodium channels and influx as compared to higher temperatures, increasing the vulnerability of the nervous system to the toxic effects of this compound (Jegede et al., 2017).

The fungicide Score 250 EC® had no effects on *F. candida* at both temperatures tested (23 and 33 °C). Oliveira et al. (2018) determined an EC_{50} for *F. candida* of this compound of 28.9 mg a.s./kg soil. *F. candida* was clearly less sensitive to difenoconazole than to abamectin. Given the mode-of-action of these compounds, collembolans may indeed be expected to be more sensitive to insecticides like abamectin than to fungicides like difenoconazole (Daam et al., 2011; Oliveira et al., 2018; Pitombeira de Figueirêdo et al., 2019). The toxicity of difenoconazole to invertebrates, such as *F. candida*, remains unknown, since few studies have been carried out with this group of organisms (US Environmental Protection Agency, 2016). Regarding vertebrates, experiments with fish (*Danio rerio*) indicate that the toxic effects result from a specific mode of action and are not related, therefore, to systemic toxicity (Teng et al., 2018).

Temperature has already proven to influence the action of fungicides such as difenoconazole, as confirmed by Filimon et al. (2015), who detected a lower enzymatic activity of the soil microbiota when exposed to difenoconazole at higher temperatures. Temperature also influenced the rate of dissipation of these compounds in natural soils, as tested by Guo et al. (2010), Castillo and Torstensson (2007) and Fenoll et al. (2009), supporting our results, especially from tests at higher temperatures.

For the mixture of the compounds, the statistical analysis performed (estimated values, Table S2) confirmed that the toxicity is mainly due to the insecticide Kraft 36 EC®. Since we tested only one combination of the pesticides, it is not possible to show synergistic, additive or antagonistic effects.

3.2. Sensitivity of the plants to the pesticides

The effects on both plant species tested were significant, especially for dry weight and length at 23 °C, while at 33 °C an increase in weight (dry weight) and length was observed for both plant species. For fresh weight, temperature increase, as an isolated parameter, was the most significant parameter, making it impossible to verify the effect of the contaminants alone or in mixture (Tables S3 and S4; Figs. 2 and 3).

Tiyagi et al. (2004) found adverse effects on plant growth parameters (plant height, weight and root nodulation) after chickpea plant exposure to aldicarb, carbofuran, phorate, fensulfothion, and fenamiphos. However, in the present study the fungicide Score 250 EC® caused

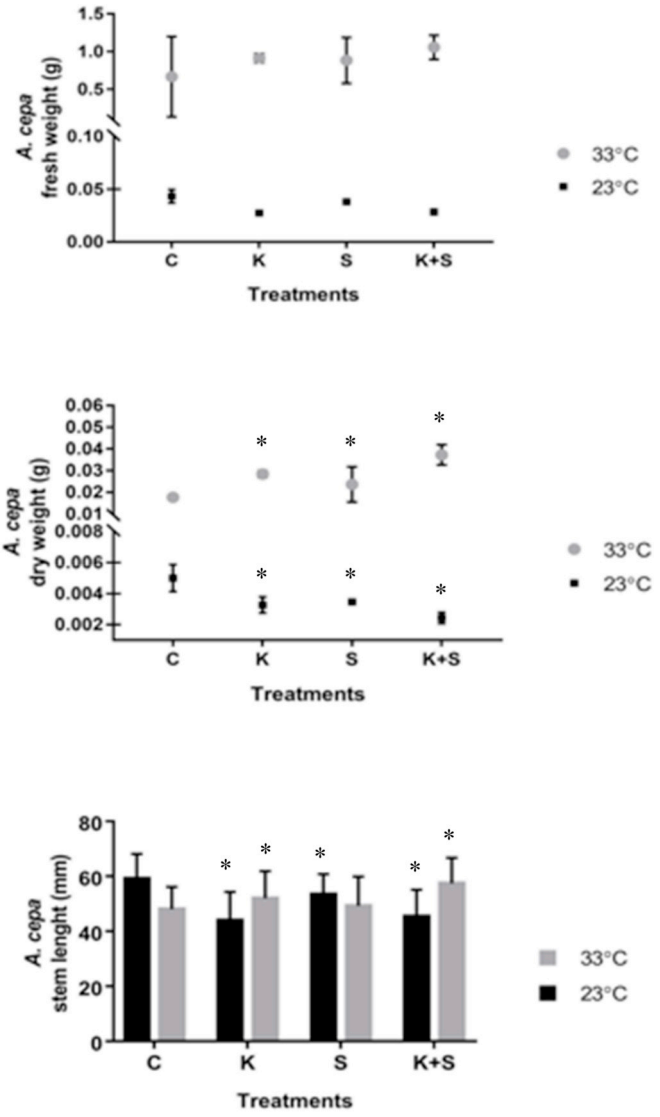

Fig. 2. Mean ± standard deviation fresh weight (g), dry weight (g) and length (mm) of *Allium cepa* grown for 18 days at 23 °C and 33 °C in mesocosms non-treated (C- 8 control) or treated 3 times at 7-day intervals with normal doses of abamectin in the formulation Kraft (K), difenoconazole in the formulation Score (S) or their binary mixture (K+S). Asterisks (*) mean statistical difference from control (GLM, $p < 0.05$).

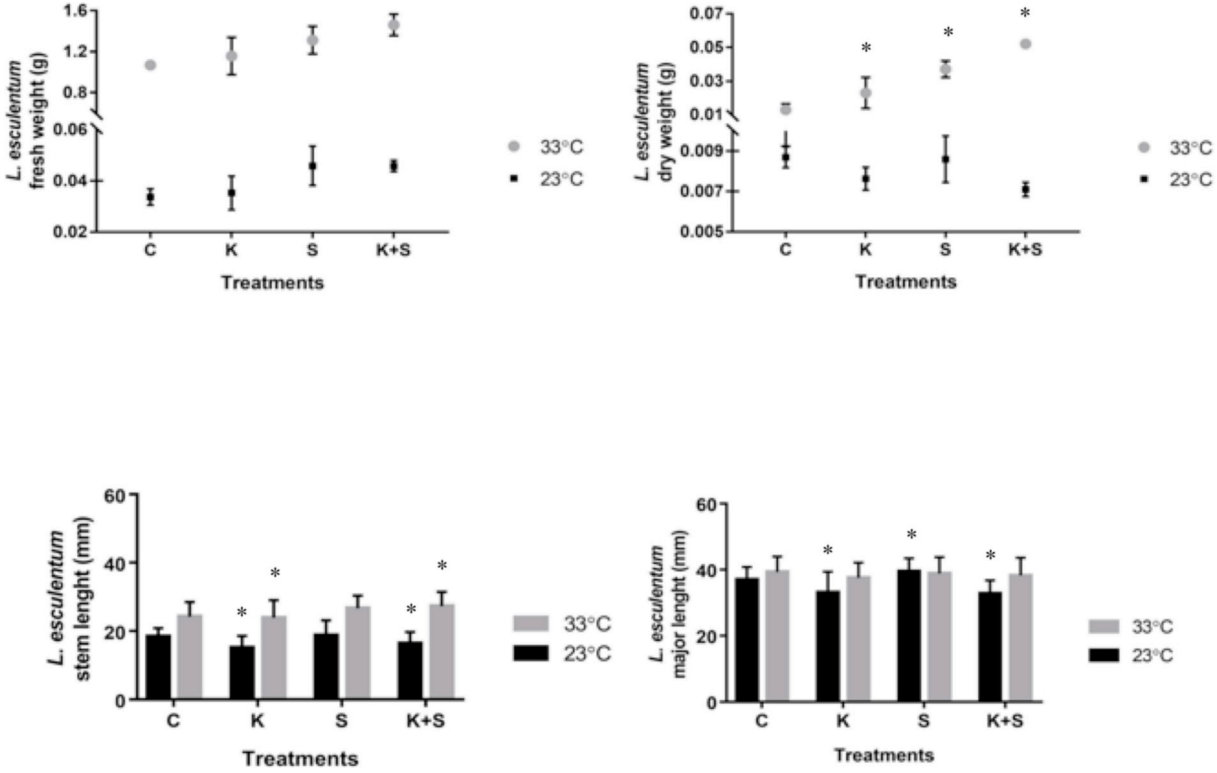

Fig. 3. Mean ± standard deviation fresh weight (g), dry weight (g) stem length (mm) and major length (mm), of *Lycopersicum esculentum* grown for 18 days at 23 °C and 33 °C in mesocosms non-treated (C- control) or treated 3 times at 7 day intervals with normal doses of abamectin in the formulation Kraft (K), difenoconazole in the formulation Score (S) or their binary mixture (K+S). Asterisks (*) mean statistical difference from control (GLM, $p < 0.05$).

an increased dry and fresh weight, specially at 33 °C, of both plant species (Figs. 2 and 3). This corroborates with Grossmann (1990) who demonstrated that triazoles pesticides influence the cytokinin content that could allow an increased plant biomass by killing pathogenic mesoorganisms (Van Den Brink et al., 2000). An increase in germination, plant height, and dry weight was also found by Mohamed and Akladious (2017) when studying the effects of the fungicides Maxim (fludioxonil), Vitavax T (carboxin and thiram), Hemixet (hymexazol) and Flosan (thiram) used on cotton.

Ahemad and Khan (2012) also showed that four fungicides (tebuconazole, hexaconazole, metalaxyl and kitazin), two of them of the same class of fungicide studied in this work, at recommended doses, had less reducing effects on plant growth promoting factors. Baćmaga et al. (2016) also found significant effects of the pesticide Falcon 460 EC® (fungicide, containing active ingredients spiroxamine, tebuconazole and triadimenol) on seed germination capacity and root elongation of *Lepidium sativum, Sinapsis alba* and *Sorghum saccharatum* at concentrations 30 times greater than the recommended dose. However, in the same study the authors reported strong inhibition of the activity of dehydrogenase, catalase, urease, acid phosphatase and alkaline phosphatase.

The monocotyledonous species *A. cepa* was more sensitive to the studied pesticides than the dicotyledonous species *L. esculentum* (Figs. 2 and 3). This is confirmed by Boutin et al. (2004) who suggests that the anatomical and physiological differences of the plant types make them respond differently to pesticide toxicity.

Temperature seemed to affect seed germination, especially of *A. cepa* with about 50% less germination at the higher temperature (Table 1). However, it is already known that increasing the temperature alters the physiological activities of the plant and may even lead to dormancy at very low (0 °C) or high (25–30 °C) temperatures (Resende et al., 2007). The plant weight and length suggested a reduced toxicity with increasing temperature (Figs. 2 and 3). This can be explained by

Table 1
Total number of germinated plants in mesocosms nontreated (Control; C) or treated 3 times at 7-day intervals with normal doses of abamectin in the formulation Kraft (K), difenoconazole in the formulation Score (S) and the binary mixture of Kraft and Score (K + S) for the species *Allium cepa* and *Lycopersicum esculentum* in the experiments performed at 23 °C and 33 °C of a total of 45 plant seeds for each species.

	A. cepa		*L. esculentum*	
	23 °C	**33 °C**	**23 °C**	**33 °C**
C	38	15	31	28
K	32	14	33	28
S	34	16	35	29
K+S	32	18	33	29

the degradation of the most toxic compound studied, abamectin, which corroborates the results of Boukhrissa et al. (2017) who proved that abamectin degradation (Kraft 36 EC®) increases with increasing temperature. This antagonistic behavior of temperature and toxicity was also found by Lima et al. (2015) when studying the effects of carbaryl on *Triticum aestivum*. However, in the same paper the authors also report synergistic effects of this chemical with temperature on another species (*Brassica rapa*) demonstrating this relationship is species specific.

In the evaluation of combined pesticides much has been studied about their effects on a wide range of organisms, especially for binary mixtures. Martin and Ronco (2006), when studying a cypermethrin-glyphosate mixture, found a 7 times higher toxicity of the mixture compared to the individual components on root elongation of *Lactuva sativa* L., corroborating with Cedergreen (2014) who affirms that for autotrophic organisms the main effect of pesticide mixtures is synergism. However, as in this study, there was no difference in the toxicity

of the mixture of the compounds and that of the individual pesticides, since the mixture effect was dominated by that of the insecticide abamectin (Kraft 36 EC®) for all evaluated parameters. Queirós et al. (2018) also reported no additional effect of a combination of terbuthylazine and nicosulfuron for *Portulaca oleracea*, stating that adding nicosulfuron apparently is useless.

4. Conclusions

The insecticide abamectin, formulated as Kraft 36 EC®, is extremely toxic to the collembolan *F. candida* and to the plant species *A. cepa* and *L. esculentum*, with strong effects already seen at the recommended dose. Effects were highest at 23 °C, which indicates a high environmental risk of this compound, since this pesticide is mostly applied in temperate regions with temperatures around 20 °C. At the higher test temperature, pesticide degradation leads to a reduced toxicity for the species evaluated in this study. The fungicide difenoconazole was not toxic to the tested species at recommended field dose.

CRediT authorship contribution statement

Livia Pitombeira de Figueirêdo: Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing. **Danillo B. Athayde:** Methodology. **Michiel A. Daam:** Conceptualization, Writing - review & editing. **Cornelis A.M. van Gestel:** Writing - review & editing. **Glauce da Silva Guerra:** Methodology. **Paulo José Duarte-Neto:** Methodology. **Evaldo L.G. Espíndola:** Conceptualization, Resources, Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110446.

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