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Short Communication

Effects of cycloheximide on the mortality of *Atta sexdens* leaf-cutting worker ants

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ABSTRACT

Leaf-cutting ants live symbiotically with a fungus that they cultivate on the plant leaves that they cut. The innumerous studies on the plant selection mechanism used by leaf-cutting ants show the researchers' interest in this issue. Many classical studies propose that plants are selected according to the fungus garden nutritional needs and the absence of potentially harmful substances. This hypothesis is corroborated by behavioral experiments using cycloheximide (fungicide) with citric pulp or forage plants greatly accepted by leaf-cutting ants. According to this hypothesis, under the action of a fungicide, the fungus emits an allomone that informs worker ants that some food is inadequate to its growth. Although some authors state that the cycloheximide "fungicide" used is specific and non toxic to ants, our findings are distinct. In our study, various concentrations of cycloheximide were administered orally to leaf-cutting worker ants in a citric pulp paste diet. After the ingestion period, the ants were isolated and offered the symbiotic fungus for 21 days and the mortality rate was evaluated. As expected, the treatment with 0.01% cycloheximide showed a low mortality rate (8.86%). At 0.1%, the mortality rate was mild (27.85%), and treatment with 1% cycloheximide resulted in moderate mortality (45.57%). In contrast, the positive control with 0.1% sulfluramid showed a high mortality rate (91.14%). Therefore, we concluded that the ingestion of high concentrations of cycloheximide results in a moderate mortality rate in leaf-cutting worker ants.

Leaf-cutting ants (LCA), insects from the Neotropical region, are known mainly as a pest in some cultures, but also due to their mutualism with the symbiotic fungus *Leucocoprinus gongylophorus* (Heim) Moeller, the colony food source (Hölldobler and Wilson, 1990; Schultz et al., 2005). Many classical studies propose that plants are selected according to the fungus garden nutritional needs and the absence of potentially harmful substances (Britto et al., 2016). This hypothesis is corroborated by behavioral experiments using cycloheximide (CHX), known to inhibit protein synthesis; however, its mechanism of action is still unknown. It is also a known "fungicide" in studies with LCA, especially in behavioral studies on fungus-ant communication toward plant selection. The first studies reported that CHX modifies the foraging behavior by delaying its recognition in LCA workers, which do not identify it as harmful to the colony in their first contact with it. However, soon afterwards, the incorporation of CHX is interrupted. This behavior has been attributed to communication between the fungus and LCA workers through a volatile semiochemical emitted by the fungus (Ridley et al., 1996).

Additionally, behavioral studies report the hypothetical existence of fungus-ant communication through a volatile semiochemical yet to be identified and learning processes (Ridley et al., 1996; North et al., 1999). Recently, Sousa et al. (2017) observed an alteration in the foraging and fungus garden cultivation behaviors of LCA workers offered citric pulp pellets with CHX in laboratory. The authors reported a significant increase in mortality of workers in waste chamber, suggesting a potential mortality of LCA due to CHX. Many studies have been conducted with CHX to analyze the cells responsible for long- and short-term memory in insects. However, few studies have demonstrated the effect and toxicity of CHX to LCA (Akahane and Amakawa, 1983; Jaffé, 1980; Fahrbach et al., 1994; Nouri and Fallon, 1987; Wittstock et al., 1993; Matsumoto et al., 2013), except Marcos et al. (1982), who reported its toxic effect on *Drosophila melanogaster* Meigen eggs, larvae and adults with ensuing pronounced mortality, an effect to be expected, since CHX is highly toxic to many animals (Bennett et al., 1972).

Based on the above-mentioned literature, we observed the inexistence of studies on the effect of CHX on LCA workers. For that, we evaluated the effect of various concentrations of CHX given orally to LCA workers.

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Studied colonies

LCA workers from an approximately 9-year old *Atta sexdens* Forel colony kept in the Social Insect Laboratory (LISP) in UNESP, Botucatu, São Paulo, Brazil were used in this study. The colony is housed in 13 containers with nine fungus garden chambers with a 2-cm plaster of Paris layer to preserve fungus chamber moisture and two forage chambers and two waste chambers. Each chamber is 18 cm in diameter and height. The chambers are interconnected by transparent plastic tubes to both the forage and waste chambers. The colony was offered *Acalypha* spp., *Ligustrum* spp. and *Citrus* spp. leaves as substrate for the symbiotic fungus and kept at 24 ± 2 °C under relative humidity of 80% and 12-h light period.

CHX-citric pulp paste formulations

LCA worker mortality due to oral ingestion of CHX-citric pulp paste formulations was evaluated following the protocol developed by Nagamoto et al. (2004) and established by the Ministry of Agriculture, Husbandry and Supply/Pest Control Department (Ministério da Agricultura, Pecuária e Abastecimento/Secretaria de Defesa Agropecuária – MAPA/SDA) (Brasil, 2011). This study sought to identify products effective in the control of LCA and presents ant mortality rate observation methods as a result of the oral ingestion of substances. In this protocol, bait pastes prepared with powdered citric pulp, p.a. sucrose and distilled water are given to a group of LCA workers. Each experiment involved a negative control (citric pulp paste formulation), a standard or positive control (0.1% sulfluramid, [Griffin, USA]) and three CHX concentrations (CHX (Sigma–Aldrich, St. Louis, Missouri, USA) (1%, 0.1% and 0.01%), totaling five treatments in four repetitions (each repetition is a plastic pots with 20 LCA workers, according to Nagamoto et al. (2004). The concentrations used were established based on studies conducted by Ridley et al. (1996) and Sousa et al. (2017) using a CHX concentration of 1.25%.

All formulations were prepared as w/w (weight/weight). The active ingredients, sulfluramid and CHX, were first dissolved in p.a. acetone. Next, powdered citric pulp was added and the mixture was homogenized. After the complete evaporation of the solvent, this preliminary formulation was homogenized using a glass rod before adding a 10% aqueous solution of glucose to form a uniform and humid bait paste. The negative control bait was prepared in the same way without addition of the active ingredients (CHX and sulfluramid).

Preparation and use of the bait paste with the LCA workers

Transparent plastic pots measuring 7.5 cm diameter × 5.5 cm high with air-tight lids were used in the experiments. The bottom of each pot was filled with a 1-cm layer of plaster of Paris and dried for 24 h at 50 °C, after which the plaster of Paris was wetted with distilled water before 20 LCA workers with average head width of approximately 2.2 mm were introduced. The ants were left alone in isolation without fungus and substrate for 24 h, after which 2 g of bait paste were added and left for another 24 h and then removed. After the bait paste was removed, each LCA worker group was given approximately 2.5-cm^3 of fungus garden and about 20 small LCA workers, with head measuring about 0.8–1.0 mm, were introduced, as average-sized ants are not able to take care of the fungus so efficiently (Bass and Cherrett, 1995). Mortality was evaluated on days 1, 2, 3, 5, 7, 9, 11, 14, 17 and 21 after the bait paste had been added, following Nagamoto et al. (2004). The LCA workers' mortality rate was corrected with the Abbot formula (Abbott, 1925) to eliminate the influence of natural mortality as well as standardize the results between assays.

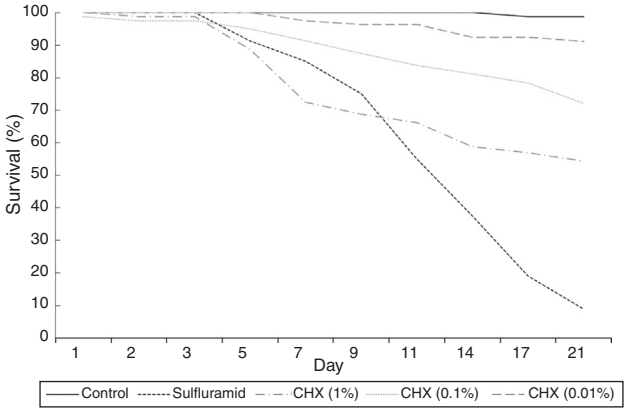

Fig. 1. Survival curves of *Atta sexdens* workers during 21 days.

Table 1
Percentage of LCA workers' mortality.

Treatments	Mortality rate (%)	
0.1% sulfluramid	91.14	a[a]
1% CHX	45.58	b
0.1% CHX	27.85	b
0.01% CHX	8.86	bc
Negative control	1.25	c

[a] Treatment sharing the same letter are not significantly different, at the 5% significance level.

Statistical analysis

First, the mortality of workers was submitted to the Shapiro Wilk test to determine whether the distribution was normal or not. After that, the mortality was submitted to variance analysis (ANOVA) (post-Tukey test) at 5% significance level.

As expected, a significant difference was detected among the various treatments for LCA workers' mortality (ANOVA, $F_{4;15} = 41.85$, $p < 0.0001$). The treatment with 0.1% sulfluramid showed high mortality rate (91.14%) and was significantly different from the negative control ($p < 0.01$), 0.01% CHX ($p < 0.01$), 0.1% CHX ($p < 0.01$) and 1% CHX ($p < 0.01$). The treatment with 0.01% CHX resulted in low mortality (8.86%), with no significant difference from the negative control. The treatment with 0.1% CHX showed low mortality (27.85%) and differed from the negative control ($p < 0.01$). However, it did not differ from the 0.01% and 0.1% CHX treatments. The treatment with 1% CHX showed the highest mortality rate (45.75%) among the CHX treatments and was significantly different from the negative control ($p < 0.01$) and 0.01% CHX ($p < 0.01$). However, the mortality rate for 1% CHX was not different from that for 0.1% CHX (Fig. 1) (Table 1 and Fig. 1).

In our study, the ingestion of CHX (in 0.1 and 1% concentrations) by *A. sexdens* resulted in an increase in the workers' mortality rate, corroborating that reported by Sousa et al. (2017). Besides, Sousa et al. (2017) observed a change in the foraging and fungus cultivation behavior when the colony was offered citric pulp pellets containing CHX, as well as degradation of the fungus garden.

We used low CHX concentrations (0.01, 0.1 and 1%) in the diet formulation in comparison to those found in the literature. For example, Sousa et al. (2017) used 1.23% CHX in citric pulp pellets offered to the colonies for seven days. Probably, the fact that the researchers increased the CHX doses offered to the colonies on consecutive days enhanced its action, and thus, the dose that the workers received was greater and so was the mortality rate. This methodology was previously used by Ridley et al. (1996) and North et al. (1999); however, they reported no LCA work mortality. Despite its identification as a protein and RNA synthesis inhibitor,

CHX has also been labeled a fungicide in studies conducted with LCA (Ridley et al., 1996; North et al., 1999). Although CHX also affects the growth of many yeasts and fungi, but has little effect on bacterium growth (Ennis and Lubin, 1964).

Another issue is the mode of action of CHX. This molecule is known to be an antibiotic produced by *Streptomyces griseus* (Obrig et al., 1971; Schneider-Poetsch et al., 2010); however, it has broad application in pioneering research. In insects, this molecule has been used in studies involving long term memory formation, resistant genotypes, morphological alteration, alteration in protein levels and destruction of neurons (Akahane and Amakawa, 1983; Flyg et al., 1980; Fahrbach et al., 1994; Oberlander et al., 1981; Soltani-Mazouni and Soltani, 1995; Nouri and Fallon, 1987). On the other hand, Marcos et al. (1982) reported a toxic effect on *D. melanogaster* eggs, larvae and adults, causing pronounced mortality in the individuals, an effect clearly expected since this substance is highly toxic to many animals (Bennett et al., 1972). CHX inhibits protein and RNA synthesis in eukaryotic organisms. Protein inhibition may reach 98% with doses as low as 1.5 mg/kg CHX; however, RNA synthesis is inhibited by doses higher than 1.5 mg/kg (Farber and Farmar, 1973). However, for LCA, as previous said, the studies do not report the mortality of worker ants and state that CHX is non-toxic to ants and that the action of the fungicide is specific (Ridley et al., 1996).

However, an LCA worker mortality rate of 46% has been observed for a high CHX concentration, which can be considered low in relation to the positive control 0.1% sulfluramid. Sulfluramid is used in Brazil as an active ingredient in the manufacture of ant baits for the control of *Atta* ("saúvas") and *Acromyrmex* ("quenquéns") LCA, the genera that cause the greatest damages to the national agriculture. The characteristics of the sulfluramid-based toxic baits currently used are: (I) delayed action in adult workers (Nagamoto et al., 2004, 2007); (II) non-repellent (Britto et al., 2016); (III) good dispersion in the colonies (Forti et al., 2007); (IV) adequate stability in the environment (Cameron, 1990); and (V) not toxic to human beings or other organisms. Additionally, the 0.1% sulfluramid affected the survival rate of workers in the period studied significantly, with a 50% reduction in the number of workers on day 14 and nearly 100% on day 21 as a result of its delayed action, an essential feature in the formulation of baits. According to Nagamoto et al. (2004), sulfluramid is a Class III insecticide with apparent indication of a broader range of concentration, close to Class IV. Insecticides are grouped in distinct formicide screening classes (Stringer et al., 1964; Lofgren et al., 1967; Vander Meer et al., 1985): (a) Class I: with a mortality rate smaller than 90% at the end of the test period, (b) Class II: compounds that kill quickly in high concentrations (mortality above 15% after 24 h and greater that 90% at the end of the test period) and cause total mortality below 90% in low concentrations, (c) Class III: delayed action compounds with mortality lower that 15% after 24 h, but above 90% at the end of the test period in a broad range of concentrations, from 1 to 9, (d) Class IV: similar to Class III, differing from those for their delayed action in a concentration range from 10 to 99, (e) Class V: rare compounds with delayed action in concentration ranges above 100.

As previously discussed, CHX cannot be considered a potential formicide because of its low mortality at high concentrations (smaller than 50%), its unknown mechanism of action in insects, lack of delayed action, in contrast to sulfluramid, and its toxicity to human beings and other organisms. In conclusion, in our study, ingestion of high concentrations of CHX promoted moderate mortality rates in LCA workers.

Conflicts of interest

The authors declare no conflicts of interest.

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