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Copper II - polar amino acid complexes: toxicity to bacteria and larvae of *Aedes aegypti*

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ABSTRACT

Control strategies using insecticides are sometimes ineffective due to the resistance of the insect vectors.In this scenario new products must be proposed for the control of insect vectors.The complexes L-aspartate Cu (II) and L-glutamate-Cu (II) complexes were synthesized and characterized by elemental analysis, visible ultraviolet, infrared spectroscopy and potentiometric titration. The toxicity of these complexes was analyzed in *Aedes aegypti* (Diptera: Culicidae) larvae and Gram-negative and Gram-positive bacteria. The interaction between the ligands and the amino acid balance and the distribution of the species as a function of pH were discussed. The lethal concentration median (LC_{50}) for *Ae. aegypti* larvae were: L-glutamic acid-Cu (II) – 53.401 mg L^{-1} and L-aspartate-Cu (II) – 108.647 mg L^{-1}. The minimum inhibitory concentration (MIC) required for *Staphylococcus aureus* and *Escherichia coli* was: L-glutamate-Cu (II) 500-2000 mg L^{-1} and L-aspartate-Cu (II) 1000-2000 mg L^{-1}. The concentrations demonstrated toxicity that evidence the potential of the complexes as bactericide and insecticide. Metal complexes formed by amino acids and transition metals are advantageous because of low environmental toxicity, biodegradability and low production cost.

Key words: Neurotransmitter, bacteria, population control of vectors, metal-insecticide, mosquito.

INTRODUCTION

The control of insect vectors of neglected diseases (dengue, chikungunya, zika) has been hampered by the occurrence of insect resistant populations, the adaptability of insects to synthetic and biological insecticides and the absence of new and effective molecules with low environmental impact (Corbel et al. 2016).

The Cu (II) ion is a bioactive metal and essential micronutrientwith a narrow range of biological toxicity that, coordinated with ligands, is transported to the intracellular environment and causes *in situ* oxidative stress reactions with the

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production of free radicals and oxidant species (ROS), as well as disorganization of the peritrophic matrix and destruction of the intestinal microvilli of mosquito larvae (Arruda et al. 2010, Gopinathan and Arumugham 2014, Nardeli et al. 2014, Gaban et al. 2015). In addition, it is a powerful antimicrobial agent that if used as a contact surface for metal alloys, inactivates microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Neciosup et al. 2015).

The glutamic acid and aspartic acid are neurotransmitters and act on the central and peripheral nervous system of insects and may be metal binders. L-glutamate (excitatory) and γ-amino butyric acid (inhibitor) are neurotransmitters that control the permeability of chloride ions (Cl^-) and the change in this interaction may lead to greater permeability to Cl^- ions, causing nervous hyperexcitation (Beyenbach 2012), which is metabolically important because it causes an increase in the metabolic activity of oxidative stress induced by the presence of transition metals. Thus, the control of immature stages of insects can be proposed by the action of metallic complexes in the digestive system and neuromuscular junction (glutaminergic action). Homeostasis is obtained by dissociation complexes with a balance of L-glutamate and γ-amino butyric acid and by changing the availability of chloride ions, Cl^-. This change affects the permeability of chloride ion junction and may block or increase nerve stimulation (Schooley et al. 2012). These conditions are unfavorable for the insect and may be promoted by the redox activity of metal complexes for induction of oxidative stress or competition for metal between important metabolic species.

The objectives of this study were the synthesis and characterization of L-glutamate-Cu (II) and L-aspartate-Cu (II) complexes for evaluation of their biological activity in *Ae. aegypti* larvae and bactericidal activity in *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) for the secondary control of the microbiota of insect breeding.

MATERIALS AND METHODS

SYNTHESIS OF COMPLEXES L-glutamate-Cu (II) AND L-aspartate-Cu (II)

Metal-amino acids were synthesized using methods available in the literature (Rayms-Keller et al. 1998, Baran et al. 2000, Brumano 2008). The studies for the two acidic α-amino acids present in almost al1proteins, i.e., L-aspartic and L-glutamic acid. The L-glutamate-Cu (II) was synthesized by mixing aqueous solutions of L-glutamic acid 19.7 mmol (Sigma-Aldrich 99%) and 19.7 mmol of copper (II) nitrate (Vetec/Sigma-Aldrich 99%) at 80°C, by stirring for 1 hour, followed by allowing the solution to stand for 24 hours in absolute ethanol to precipitate the complex L-glutamate-Cu (II). The precipitate was filtered using a filter paper, washed with absolute ethanol and then dried in vacuum desiccator for characterization. The same procedure was used for the synthesis of L-aspartate-Cu (II), 10.2 mmol of L-aspartic acid (Sigma-Aldrich 99%) and 10.2 mmol of copper (II) nitrate (Vetec/Sigma Aldrich 99%) at the same conditions, followed by precipitation, washing, and drying. The Cu (II) complexes were recrystallized.

The composition of both complexes was confirmed by elemental analysis.

CHARACTERIZATION OF THE METAL-AMINO ACID

The metal-amino acid complexes were analyzed in solid form by elemental analysis (CHN) and infrared spectroscopy (IR), and in solution by potentiometric titrations and UV-Vis spectroscopy. The IR analysis was performed using a spectrophotometer model JASCO FT/IR-4100, using KBr (Sigma-Aldrich 99%) with a scan range of 400 cm^{-1}-4000 cm^{-1}.

The potentiometric titrations were performed by combined pH electrode (pH Instrutherme 2000) with data analysis by Microcal Origin 6.0 software.

A standard solution of NaOH (0.300 mol L^{-1}) (Vetec/Sigma-Aldrich 99%) was used as the titrant for L-aspartic acid (0.0500 mol L^{-1}) (Vetec/Sigma-Aldrich 99%), L-glutamic (0.0500 mol L^{-1}) (Vetec/Sigma-Aldrich 99%), L-aspartate-Cu (II) (0.0500 mol L^{-1}), and L-glutamate-Cu (II) (0.0500 mol L^{-1}).

The elemental analysis of L-glutamate-Cu (II) and L-aspartate-Cu (II) were performed on Perkin Elmer model 2400 analyzer (IQ/Campinas, Brazil).

EVALUATION OF INSECTICIDAL ACTIVITY WITH *Aedes aegypti* LARVAE

Toxicity bioassays for analyzing insecticidal activity of amino acids and metal complexes were performed. For the compound L-glutamate-Cu (II), concentrations used were 50 mg L^{-1}, 200 mg L^{-1}, and 600 mg L^{-1}, and for L-aspartate-Cu (II) 200 mg L^{-1}, 500 mg L^{-1}, and 1000 mg L^{-1}. Plots of 20 3rd instar larvae of *Ae. aegypti* (Rockefeller strain) were used for each concentration in eight replicates, each in 20 mL solution. Mortality was observed after 24 hours of application. Distilled water was used as control, whereas amino acids glutamate and aspartate in aqueous solution (1000 mg L^{-1}), an organophosphate insecticide, Temephos® (1 mg L^{-1}),were used in the same amounts,with larvae of replicates,for the treatment.

EVALUATION OF BACTERICIDAL ACTIVITIES WITH *Staphylococcus aureus* AND *Escherichia coli*

The Minimum Inhibitory Concentrations (MIC) for *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were determined using the method of broth macrodilution, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS 2012). Mueller-Hinton broth supplemented with divalent cations calcium (100 mg mL^{-1}) and magnesium (50 mg mL^{-1}) was used for this purpose. The bacterial isolates were transferred to nutrient agar and incubated at 37°C for 24 hours. The density of the bacterial suspension was adjusted to approximately 108 CFU mL^{-1} as on 0.5 McFarland scale. This suspension was diluted 1:100 with sterile 0.85% saline and subsequently an aliquot of 20 uL was inoculated into each well (104 CFU/well). Serial dilutions were made and concentrations of the metal complexes were added to the wells. The growth inhibition was analyzed after an incubation period of 24 hours at 37°C, by observing the amino acid solutions and tetracycline antibiotic solution separately.

STATISTICAL ANALYSIS

Mortality data for *Ae. aegypti* were used to estimate the lethal concentration 50% (LC_{50}) and 99% (LC_{99}) for the larval population. The calculation of LCs was performed using the Probit analysis (StatPlus Software 2009). LCs were expressed in milligrams per liter (mg L^{-1}). Estimates of variation of LC_{50} and LC_{99} between the complexes were compared by analysis of variance (Kruskal-Wallis H) and Mann-Whitney tests. In the analysis of variance, the results of bioassays tested the null hypothesis, that the estimated LC_{50} and LC_{99} of each complex were equal, against the alternative hypothesis that at least one of the means for each parameter was different, establishing the significance level of 5%.

RESULTS

SYNTHESIS AND CHARACTERIZATION OF L-glutamate-Cu (II) AND L-aspartate-Cu (II) COMPLEXES

The complexes synthesized were obtained with yields of 70.2% for L-glutamate-Cu (II) and 80.5% for L-aspartate-Cu (II). The purification of the complexes was done by solubilizing them in water, precipitation, washing, and drying with absolute ethanol followed by slow evaporation and/or cooling. The Cu (II) complexes were recrystallized and the crystals formed were isolated, washed and the stoichiometric estimated by elemental analysis. Thus, for the complexes obtained in crystalline state it was possible to estimate from the elemental

analysis data that the purity is close to 98%. These results are summarized in Table I, which are in agreement with the general formula: $Cu(L)_2 \cdot 2H_2O$, where L= glutamate or aspartate.

The infrared spectrum of the L-aspartate-Cu (II) and L-glutamate-Cu (II) showed that the most important for the analysis bands in the spectra are carboxylate (COO^-) and amino groups (NH_2). The spectra showed a significant shift, suggesting a possible role in the coordination of the carboxylate metal-ligand (Baran et al. 2000). The resulting Vas (-COO^-) and Vs for L-aspartate-Cu (II) were 1585/1667 and 1407 cm^{-1}, respectively, and for L-glutamate-Cu (II) 1384/1400 and 1588 cm^{-1}, respectively (Figure 1).

Table II shows the displacements of the bands of carboxylate groups (COO-) for the amino acids L-glutamic acid and L-aspartic acids and metal complexes L-glutamate-Cu (II) and L-aspartate-Cu (II).

The potentiometric titration allows the analysis of deprotonation of the ligand, formation of the complex, and obtaining the constants. The data allow to predict the behavior of these species in the insect digestive tract, as at certain pH, the coordination between ligand and metal varies in the presence or absence of electrical charge that differs according to the pH of the medium in which the complexes are present. The pKa is the pH at which 50% of coexisting species are unionized (or ionized by the second time). The analysis of pKa is important for determining the coordination and distribution of species-coordinating function of pH. The tabulated values of pKa for aspartic acid are pKa1 (α-COOH) 2.01, pKr (β-COOH) 3.90 and pKa3 (-NH_3^+) 9.90, and for glutamic acid are pKa1 (α-COOH) 2.10, 4.07 pKr (γ-COOH) and pKa2 (-NH_3^+) 9.47. The pKa values determined for L-aspartate-Cu (II) were pK1 (α-COOH) 3.49, pKr (β-COOH) 3.83 and pK2 (α-NH_3^+) 8.31, and for L-glutamate-Cu (II) were pK1 (α-COOH) 5.30, pKr (γ-COOH) 6.37, and pK2 (α-NH_3^+) 9.76, respectively.

TABLE I
Theoretical and experimental values from elemental analysis for the L-glutamate-Cu (II) and L-aspartate-Cu (II) complexes.

Complexes	%C	%C *	%H	%H*	%N	%N*	%O	%O*	%Cu	%Cu*
Glutamate	26.87	24.54	3.55	3.71	6.65	5.72	-	39.24	-	25.97
Aspartate	22.20	20.82	3.63	3.94	6.65	6.07	-	41.61	-	27.55

* Theoretical value.

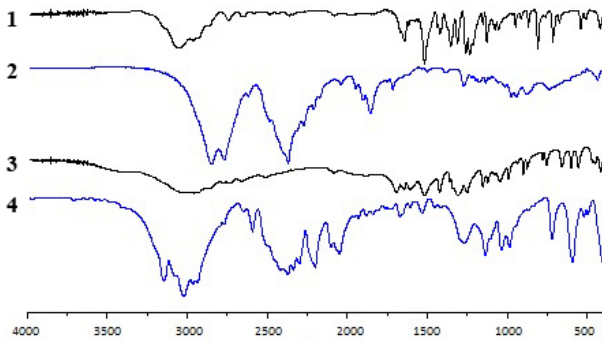

Figure 1 - Infrared spectrum of L-glutamate (**1**), L-glutamate-Cu (II) (**2**), L-aspartate (**3**) and L-aspartate-Cu (II) (**4**).

TABLE II
Caraboxylate band shifts for L-glutamic acid, L-aspartic acid and metal-amino acid complexes L-glutamate-Cu (II) and L-aspartate-Cu (II), the displacements were compared as described by Baran et al. 2000.

Samples	$V_{as}(COO^-)$ (cm^{-1})	$V_s(COO^-)$ (cm^{-1})
L-aspartic acid	1607, 1560	1421
L-glutamic acid	1665, 1639	1436, 1419
L-aspartate-Cu (II)	1667, 1585	1407
L-glutamate-Cu (II)	1588	1400, 1384

The results of the potentiometric titration permit analysis of pKa and the balance and distribution of species of the complexes L-aspartate-Cu (II) and L-glutamate-Cu (II) as a function of pH (Figure 2).

Species equilibrium for aspartic and glutamic acids are shown in Figures 3 and 4.

The results of potentiometric titration, visible ultraviolet, and infrared spectroscopy suggest that greater absorption of L-glutamate-Cu (II) occurs in the midgut. The midgut of the insect has a pH of 10,at which glutamic acid is released with all hydrogen ions and is neutralized and the metal-amino acid complex carries no charge, which allows greater permeation in the digestive system through the cell membrane. However, due to system complexity, it is not confirmed that the midgut is the only permeation site for metal-amino acid complex absorption. Figure 5 shows the possible structural arrangement of L-glutamate-Cu (II) complex at pH 10.

EVALUATION OF INSECTICIDAL ACTIVITY USING *Aedes aegypti* LARVAE

After 24 hours exposure of 3rd instar larvae to the metal complex solution, the values found for the concentration-mortality by L-aspartate-Cu (II) were: 1000 mg L^{-1} (100%), 500 mg L^{-1} (95%); 200 mg L^{-1} (76.66%), and for L-glutamate-Cu (II) were 600 mg L^{-1} (83.18%), 200 mg L^{-1} (64.54%), and 50 mg L^{-1} (47%). In the control, 100% mortality occurred only for the insecticide Temephos® 1 mg L^{-1}. These results showed that the mortality curves for the complex are concentrated in the upper 50%,thus indicating that the concentrations used in the bioassays were high and the lethal concentrations (LC) obtained could be reduced (Table III). The concentrations used in toxicity bioassays for each metal-amino acid complex are different. However, for higher concentrations the mortality percentages are similar, which is not true for the other concentrations diluted, possibly due to the solubility of the complexes.

LC for L-glutamate-Cu (II) are in the confidence interval of L-aspartate-Cu (II), demonstrating that the effect of the insecticide on both metal complexes is similar and that the amino acid ligands have no impact on mortality of *Ae. aegypti*. Statistical comparisons confirmed bioassays with significant differences ($p<0.05$) in mortality, suggesting that, despite the low concentrations of L-glutamate-Cu (II), the insecticidal activity was similar. Statistical analysis showed that it is not possible to state that the L-glutamate-Cu (II) is superior to L-aspartate-Cu (II), as observed from the analysis of the concentrations of 1000 mg L^{-1} and 600 mg L^{-1} after 24-hours exposure. The lethal concentration LC_{50} and LC_{99} were obtained through the POLO-PC program, and the LC_{50} is important as at this concentration, the standard deviation is lower and most of larval population is studied at LC_{50}.

EVALUATION OF BACTERICIDAL ACTIVITY USING *Staphylococcus aureus* AND *Escherichia coli*

The MIC for *S. aureus* ATCC 25923/L-glutamate-Cu (II) was 500 mg L^{-1} and *E. coli*/ATCC 25922/L-glutamate-Cu (II) was 2000 mg L^{-1}, and

TABLE III
Lethal Concentrations (LC) (mg L^{-1}) and confidence interval at 95% ($IC_{0.05}$) complex of L-aspartate-Cu (II) and L-glutamate-Cu (II) obtained with the mortality of *Ae. aegypti*.

Complexes	LC_{50} ($IC_{0.05}$)	LC_{99} ($IC_{0.05}$)	b±SE*	χ_2	df**
L-aspartate-Cu (II)	108.647 (62.106 – 144.984)	809.782 (568.044 – 1534. 645)	0.485±1.189	4.496	22
L-glutamate-Cu (II)	53.401 (31.124 – 94.546)	437.881 (205.178 – 947.357)	1.241±1.543	3.247	22

* b ± SE Coefficient Angle ± Standard error; ** df Degrees of Freedom.

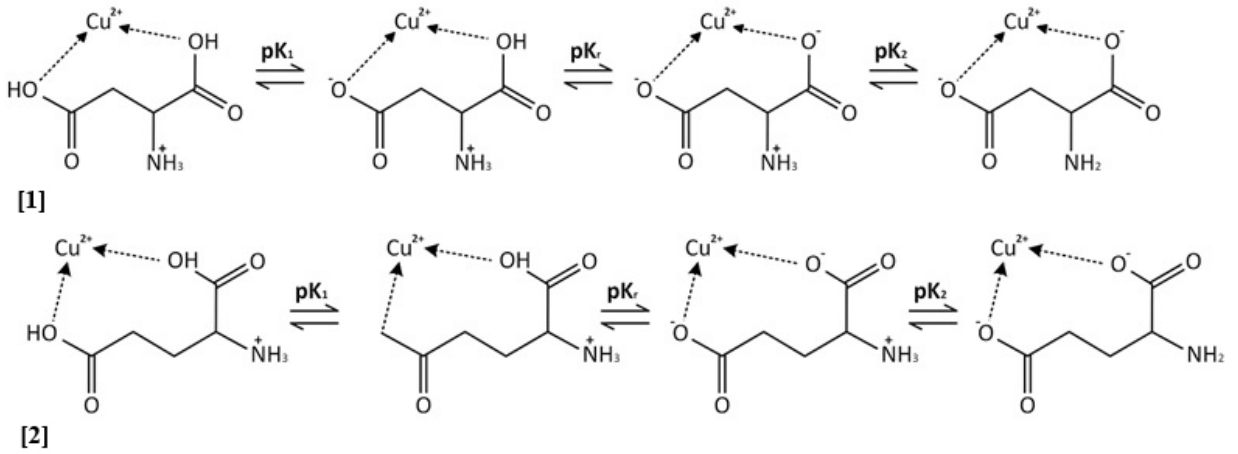

Figure 2 - Structural forms: L-aspartate-Cu (II) [**1**] and L-glutamate-Cu (II) [**2**] in aqueous medium.

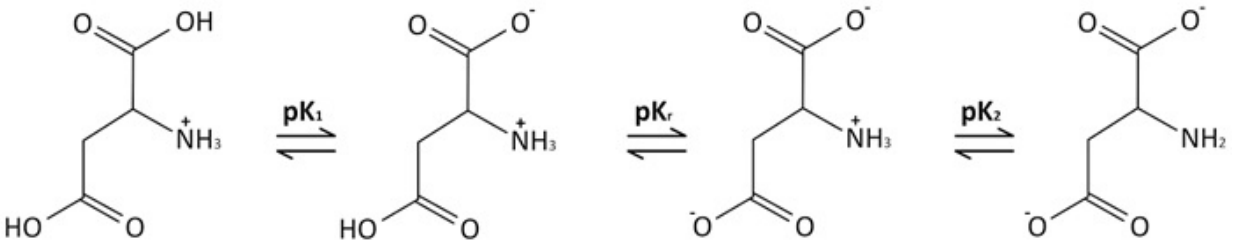

Figure 3 - L-aspartic acid equilibrium in aqueous medium.

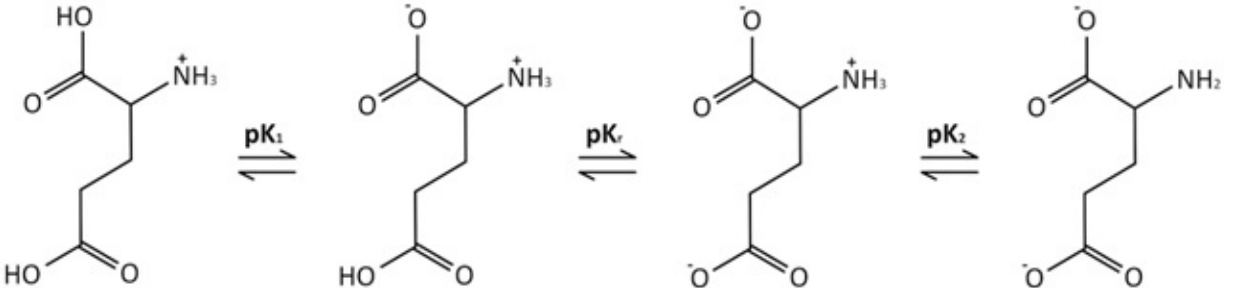

Figure 4 - L-glutamic acid equilibrium in aqueous medium.

for *S. aureus* ATCC 25923/L-aspartate-Cu (II) was 1000 mg L^{-1} and *E. coli* ATCC 25922/L-aspartate-Cu (II) was 2000 mg L^{-1}. The results showed that the metal complexes have different biological activity for Gram-positive (*S. aureus*) and Gram-negative (*E. coli*). This aspect could be related to formation of the bacterial cell wall. Gram-positive bacteria have a single wall that consists of peptidoglycan, whereas Gram-negative bacteria have three walls that are composed of polysaccharides, phospholipids and peptidoglycan. These different characteristics in the constitution of the cell wall results in increased resistance to antibiotics in Gram-negative bacteria (Ponnusamy et al. 2008), due to which MIC for *E. coli* is higher. Bacterial growth was not inhibited in the control.

DISCUSSION

The potentiometric titration demonstrated the equilibrium and the distribution of the metal

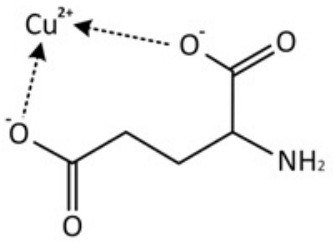

Figure 5 - Complex L-Glutamate-Cu (II) at pH 10 with neutral electric charge.

complex solution in the pH range of 5 to 10, being the pH of absorption and excretion of the metallic complexes in the digestive tract of the insect. The pH analysis also allows the understanding of metal-amino acid coordination, species distribution, toxicity and the electrical charge capacity of the metal to affect the toxicity of the amino acid-Cu (II) complex (Sajadi 2010).

Insects have a complete digestive tract, being: mouth, anterior, middle and posterior intestine (Matta et al. 2016). In this system there is difference of pH being that at pH 10 occurs the absorption of substances in the middle intestine. Gaban et al. (2015) showed that EDTA-Cu (II), at the concentration of 125 mg L^{-1}, causes brush edge damage and increased cytoplasmic vacuoles in gastric and midgut cells, leading to rupture of the cell junction regions of the stomodeus, possibly due to pH dependent oxidative stress reactions. At pH 10, the ligand is deprotonated to acquire a neutral charge, thereby indicating increased permeation of the complex through the cell membrane.

The toxicity of the metal-amino acid complexes may be related to the lipid solubility of the molecule, since the cell membranes possess a lipid bilayer responsible for the cellular permeation mechanism through the Na^+ and K^+ ion channels (Vasconcelos et al. 2007, Ponnusamy et al. 2008). L-glutamate has higher lipid solubility than L-aspartate and this difference in solubility can increase the efficiency of cellular permeation with consequent increase in the transport of the metal complexes, facilitating the induction of oxidative stress reaction in the extracellular environment and the intracellular environment.

The bactericidal effect suggests that the metal complexes can act in a comprehensive (multifunctional) way not only on the target organisms (mosquito larvae), but also on all the bacterial microbiota installed in the mosquito breeding sites. The larvae would come in contact with the metallic complex from the hatching, passing the entire larval stage in that condition. The decrease of the bacterial microbiota would cause food shortages, helping in larval mortality and consequently of the adults that developed in that breeding place.

The results obtained in this study are based on laboratory studies of bioactive substances containing Cu (II) as active ingredient. Commercial products have not yet been produced and further studies are needed to determine the stability of the metal complexes and their mode of action in the field. Currently, it is known that metal complexes have been used commercially, however, studies are being conducted with respect to metal encapsulation and slow release.

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