Effects of gallic acid and Zn, Cu, and Ni on antioxidant enzyme activities of Hyphantria cunea larvae infected with Bacillus thuringiensis

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Research Article

Keywords: Antioxidant enzyme, Gallic acid, Metal exposure, Hyphantria cunea, Infection

Posted Date: April 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-394796/v1

Abstract

The effects of copper, nickel, and zinc and the potent antioxidant gallic acid on the antioxidant enzyme activities of *Hyphantria cunea* larvae infected with *Bacillus thuringiensis* subsp. *kurstaki* have been identified in this study. With metal exposure, all the enzyme activities have increased. Antagonistic effects were observed in the combination of gallic acid with all three metals on the activities of superoxide dismutase and catalase. In glutathione peroxidase activity, an antagonistic effect was observed in gallic acid plus nickel group, while there was a synergistic effect for gallic acid plus zinc and gallic acid plus copper. Activities of these enzymes in larvae exposed only to the metals increased in the infected groups; while exposure to gallic acid alone elicited a decrease. As a consequence, it was found that enzyme activities were affected by both metals and gallic acid and infection.

Introduction

Insects and plants have evolved together over millions of years with the ongoing adaptation of insects to plant defense characteristics. The defense of plants against herbivorous insects may be by morphological and chemical means. The former may involve trichomes, spines, cuticles, and lignified cell walls form as first line of defense against insect pests. Chemical plant defense includes secondary metabolites such as alkaloids, phenolics, and terpenes (Lin et al. 2020). They do not participate in primary metabolic functions, but are involved in essential biological processes such as survival (Li et al. 2019). Gallic acid (GA), a secondary metabolite, is a phenolic compound present in various plants, and studies have shown that GA and its derivatives are antioxidants with anti-inflammatory and antimicrobial properties (Lu et al. 2006). Another plant defense strategy is based on metals taken up from the environment. Essential metals can be both harmful and necessary for life depending on their concentration, so their balance is essential. Metals accumulated in plants have been shown to cause harmful effects on insect herbivores (Cheruiyot et al. 2015). When cells use oxygen to generate energy, free radicals are formed. These radicals are usually reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) resulting from cellular redox processes. At low to moderate concentration, ROS have beneficial effects on cellular responses and immune function but may cause oxidative stress at high concentrations, damaging cell structures such as proteins, DNA, and lipids (Pham-Huy et al. 2008). All aerobic species have antioxidant defense mechanisms to balance the destructive effects of free radicals. Antioxidant enzymes involved are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). Although SOD reduces the superoxide radical to hydrogen peroxide, H_2O_2 generated by SOD is reduced to water by CAT and GSH-Px (Surai 2016). The fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) is a worldwide pest which causes critical crop damage in Turkey and many other countries. Various control methods have been used to prevent damage caused by this species including biological control, one of the most widely used being *Bacillus thuringiensis* Berliner (Lacey et al. 2001) was used. Insects are excellent model organisms for studies about the effects of environmental pollution on the immune system (Pölkki et al. 2012). In this research, Cu, Ni, and Zn have been included, which are essential in

trace amounts but may cause environmental pollution at high concentrations. Since GA is a potent antioxidant, the effects of these metals and GA both alone and in combination on the antioxidant enzyme activities of larvae infected by *B. thuringiensis* subsp*. kurstaki* (*Btk*), widely used as insecticide.

Materials And Methods

Obtaining larvae and preparing artificial diets

H. *cunea* larvae were collected from the Çarşamba District of Samsun Province of Turkey in 2020. The larvae brought to the laboratory were kept at 25 ± 2 °C and 70 % humidity (16 hours light/8 hours dark) and were fed with the diet developed by Yamamoto (1969) until they reached the pupal stage. The larvae of the 2nd generation obtained from the 1st generation were used for the experiment. GA, Zn and Cu used in the study were purchased from Sigma-Aldrich (Darmstadt, Germany) and Ni was purchased from Merck (Darmstadt, Germany). Different diets were prepared by adding GA as secondary metabolite and Zn, Ni, and Cu to the control diet. A total of eight diets were prepared: one diet by adding 1 g L^{-1} GA to artificial diet (Yamamoto 1969), three diets adding 1.5 g L^{-1} of zinc, nickel, and copper, and three by adding 1 g L^{-1} GA and 1.5 g L^{-1} of each metal individually; one was the control diet group.

Bacteria and culture conditions and infection of larvae with bacteria

Btk was used in larval infection. The strain was obtained from culture collection of microbiology laboratory at Karadeniz Technical University. The *Btk* was grown overnight at 30 °C in nutrient broth (AppliChem, Darmstadt, Germany). The optical density of the growing culture was measured at a wavelength of 600 nm and set to OD_{600} = 1.89 (Danismazoglu et al. 2012). For infected groups, 1 mL of the bacterial suspension at this density was sprayed onto artificial diets.

Experimental setup

Each group consisted of hundred larvae *H. cunea*. The larvae in the control groups were fed control diet for five days, and then the hemolymph of the larvae were taken by cutting third legs of the larvae. After five days, 1 mL of *Btk* suspension was sprayed into the diet of the larvae to be infected and continued to be fed for two more days. Then, the hemolymph of the larvae was taken, and enzyme analyses were performed.

Enzyme analysis

The hemolymph samples taken from the larvae were homogenized with an ultrasonic processor (VCX 130 Sonics, Newtown, CT, USA). The homogenates, 20 mL each, were centrifuged for 20 minutes at 15000 rpm a refrigerated centrifuge (model 3500, Kubota, Tokyo, Japan). Protein in the supernatants was determined according to Lowry et al. (1951). For this method, the intensity of the color caused by amino acids in the side chain of the reduced copper and proteins by reducing the Folin-Phenol reagent was measured spectrophotometrically at 595 nm. Activities of SOD were determined according to McCord and

Fridovich (1969) and Flohé and Ötting (1984), CAT according Lück (1963), and GSH-Px according to Lawrence and Burk (1976). To determine the SOD activity, the reduction of cytochrome c by the xanthine/xanthine oxidase system was spectrophotometrically measured at 550 nm. CAT activity was determined spectrophotometrically with the decrease in 240 nm absorbance due to H_2O_2 degradation. The GSH-Px activity was measured spectrophotometrically at 340 nm under the glutathione reductase cofactor and NADPH in the reaction medium. A UV/Vis spectrophotometer (model T70, Pharma Test Apparatebau, Hainburg, Germany) was used to determine enzyme activities.

Statistical analyses Two independent samples t-test was used to determine the relationship between the enzyme activities depending on the diet content in the study. The SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used for these tests.

Results

Superoxide dismutase activities

SOD activities among the control groups were 150 ± 0.6, t = 22, $p < 0.001$ at control diet, 206 ± 0.6, t = 22, $p < 0.001$ at 1.5 g L^{-1} Zn, 184 ± 1.1, t = 33, $p < 0.001$ at 1.5 g L^{-1} Cu, 157 ± 1.1, t = -9, $p < 0.001$ at 1.5 g L^{-1} Ni, 179 ± 1.4, t = -7, $p < 0001$ at 1 g GA, 116 ± 0.8, t = -17, $p < 0.001$ at GA+Ni, 121 ± 0.8, t = 7, $p < 0.001$ at GA+Cu, and 193 ± 1.1, t = 23, $p < 0.001$ at GA+Zn, respectively. In the infected groups, the highest SOD activity was found in the 1.5 g L^{-1} Cu group (240 ± 1.3, t = 33, $p < 0.001$), while the lowest activity was in the GA+Ni group (99 ± 0.6, t = -17, $p < 0.001$) (Fig. 1).

Catalase activities

Among the control groups, the highest CAT activity was in the group containing 1.5 g L^{-1} Zn (143 ± 1.5, t = 6, $p < 0.001$), while the lowest activity was in the group with gallic acid and nickel (54 ± 0.8, t = -6, $p < 0.001$). It was determined that the highest (1.5 g L^{-1} Zn: 156 ± 1.4, t = 6, $p < 0.001$) and the lowest (GA+Ni: 49 ± 0.3, t = -6, $p < 0.001$) activities were also found in these groups (Fig. 2).

Glutathione peroxidase activities

Among the control groups, the highest GSH-Px activity was in the group in which gallic acid and zinc were used together (144 ± 0.7, t = -12, $p < 0.001$). Again among these groups, the lowest activity detected was in the control diet (77 ± 0.4, t = 16, $p < 0.001$). In the infected groups, the highest GSH-Px activity was 139 ± 1.6, t = 4, $p < 0.001$ at 1.5 g L^{-1} Zn, while the lowest one was 76 ± 0.6, t = -19, $p < 0.001$ at 1.5 g L^{-1} Ni (Fig. 3).

Discussion

Addition of 1.5 g L^{-1} each Zn, Cu, and Ni to the diet increased SOD activities compared to the control, elevating oxidative stress. In research conducted by Surai (2016), it was found that SOD activity

increased with the addition of Cu to the diet. In this study, it was determined that the groups with the highest SOD activities were the groups containing zinc and copper. Since SOD is a metalloenzyme, the presence of zinc and copper is essential for the reaction because these metals are structural and catalytic components of the SOD enzyme. GA, which shows radical scavenging ability, has significant antioxidant activity. Gallate esters show antioxidant action against superoxide radicals (Satoh et al. 1998). In current study, it was found that SOD activity increased with the addition of GA compared to the control. GA also forms stable complexes with Ni, Cu, and Zn (Masoud et al. 2012). There was an antagonistic effect on the SOD activities of the groups in which GA and metals were used together; that is; the effect of the two chemicals together was less than the effect of either one. This decrease in SOD activity proves that GA scavenges free radicals in the medium due to its powerful antioxidant properties. Pesticides can lead to the formation of free radicals by causing oxidative stress (Banerjee et al. 1999). In this study, it was found that the SOD activities of all infected groups except GA, Ni, and GA+Ni were increased compared to their controls. Also, antagonistic effects were observed in the infected GA+Ni and GA+Cu groups. This situation contradicts with the research by Masoud et al. (2012) found that GA complexes and its derivatives with Ni and Cu do not exhibit biological activity against Gram-positive bacteria *Staphylococcus aureus*.

It was also determined that the addition of all three metals to the diet has improved CAT activity compared to control. This finding may mean that these metals cause increased CAT activity by inducing oxidative stress. Li et al. (2005) showed that the application of GA restored CAT activity, which is similar to our findings that GA and metals exhibit CAT activities close to control when used together. GA is a robust chelating agent (Masoud et al. 2012); however, the Ni-formed complex still has a less stable effect compared to other metals used in the study (Hussain et al. 2013). In current research, CAT activity was the least in the control group in which GA and Ni were used together (GA+Ni). Besides, CAT activities decreased in the infected GA, Ni, and GA+Ni groups compared to their controls, and CAT activity was minimal in the infected GA+Ni group. This situation may mean that GA has a more antioxidant effect in the Ni-formed complex, resulting in minimal free radicals in the medium. The antimicrobial activity of GA against Gram-positive and Gram-negative bacteria was demonstrated in the study by Borges et al. (2012). In this study, it was observed that the enzyme activity of the group in which GA was used decreased with *B. thuringiensis*, which is a Gram-positive bacterium, infection. In addition, among the infected groups, it was found that the maximum CAT activity was in the group containing Zn; this finding is consistent with the study demonstrating that *B. thuringiensis* is tolerant to Zn (Rathnayake et al. 2009). Besides, SOD activities were parallel to CAT activities in both control and infected groups. This situation is the result of the reduction of hydrogen peroxide resulting from SOD by CAT.

In this study, it was found that GSH-Px activities of larvae increased compared to control with the addition of each metal to the diet. In this case, these metals may have caused increased GSH-Px activity by inducing oxidative stress. Also, the study showing that GA increased GSH-Px activity (Badhani et al. 2015) coincided with the result we found in this study. In GSH-Px activity, two distinct situations existed in the groups in which GA and metals were used together: an antagonistic effect was found in the GA+Ni group; while it was determined that there was a synergistic effect in the GA+Zn and GA+Cu groups. This

result revealed that GA caused different GSH-Px activities in combination with several metals, and also correlated with the finding of Nabavi et al. (2012) that GA altered the activities of glutathione. Phenolic hydroxyl groups are known to be efficient in scavenging free radicals, and the OH group in the para position to the carboxylic group is effective in antioxidant activity (Lu et al. 2006). Therefore, the three hydroxyl groups found in GA may be responsible for the antioxidant properties of this phenolic compound. Also, GA alters bacterial hydrophobicity and enhances the electron acceptor ability for Gram-positive bacteria after GA exposure, suggesting that GA is an electrophilic substance and interacts greatly with components of the bacterial surface (Borges et al. 2013). In this study, it was observed that the GSH-Px activities of the group with GA and the groups in which GA was combined with metals (except GA+Ni) decreased as a result of infection.

Conclusions

In current study, the effect of GA on antioxidant enzyme activities against ROS caused by metals was determined. Besides, it was determined the effects of bacterial infection on antioxidant enzyme activities. It was found that the enzyme activities of this insect increased with metal exposure, but generally, these activities were decreased in combinations of GA and metals. As a result, it was concluded that the antioxidant enzymes of *H. cunea* were affected by metals, gallic acid, and infection. In this context, this study will also shed light on immunological studies with different species.

Declarations

Funding: Not applicable.

Conflicts of interest/Competing interests: Not applicable. **Availability of data and material**: The data generated and/or analyzed during the current study are available from the corresponding author.

Code availability: Not applicable.

Authors' contributions: EFT contributed to the study design; and EFT, FGS, and OY perform the data analyzes. All authors helped write the manuscript.

Ethics approval: Not applicable.

Consent to participate: Not applicable.

Consent for publication: Not applicable.

Acknowledgements: We thank for Dr. Ardahan Eski from Program of Biomedical Equipment Technology, Vocational School, Bilecik Şeyh Edebali University for his contributions.

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Figures

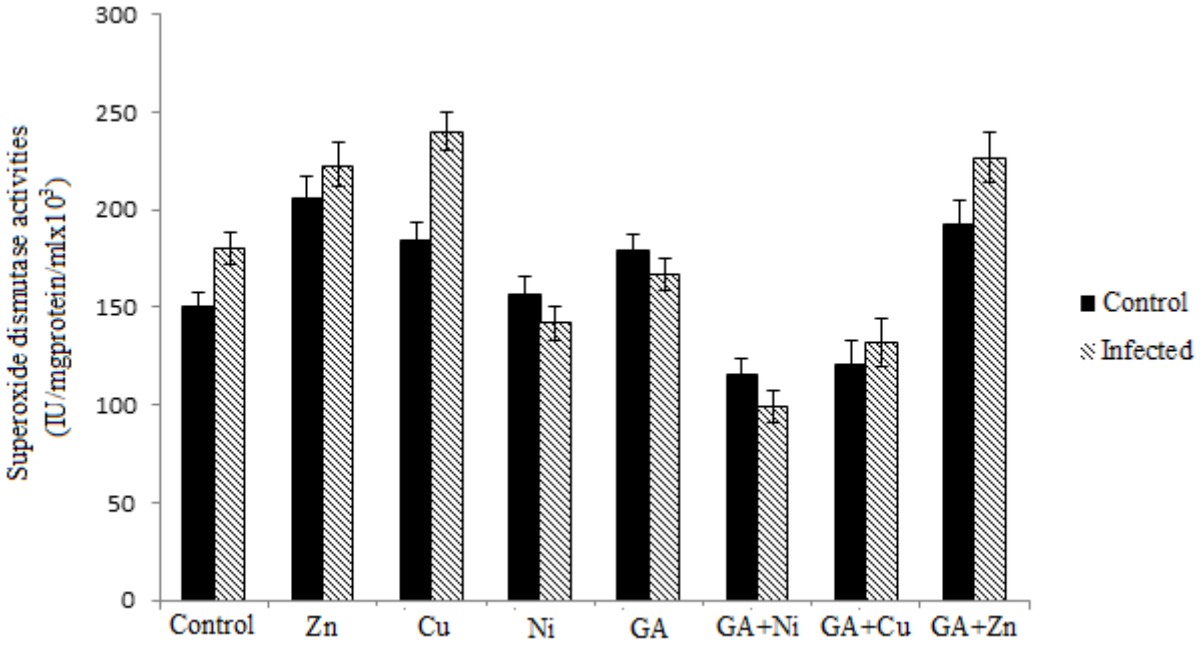

Figure 1

Comparison of superoxide dismutase activities of Hyphantria cunea larvae in the control and the infected groups. Data are expressed as mean ± S.E. Two independent samples t-test, p <0.001

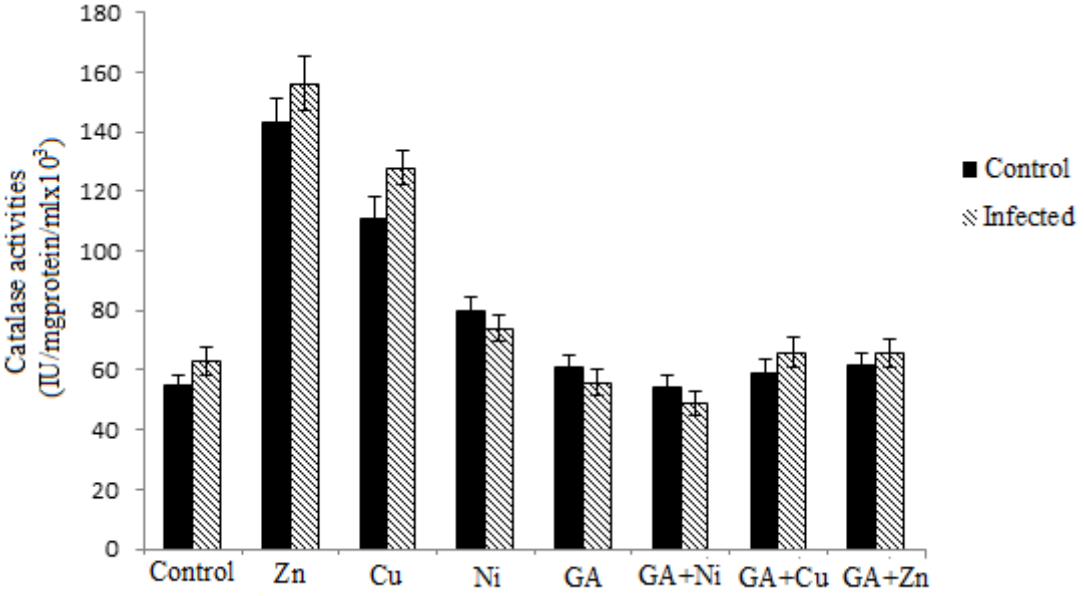

Figure 2

Comparison of catalase activities of Hyphantria cunea larvae in the control and the infected groups. Data are expressed as mean ± S.E. Two independent samples t-test, p <0.001

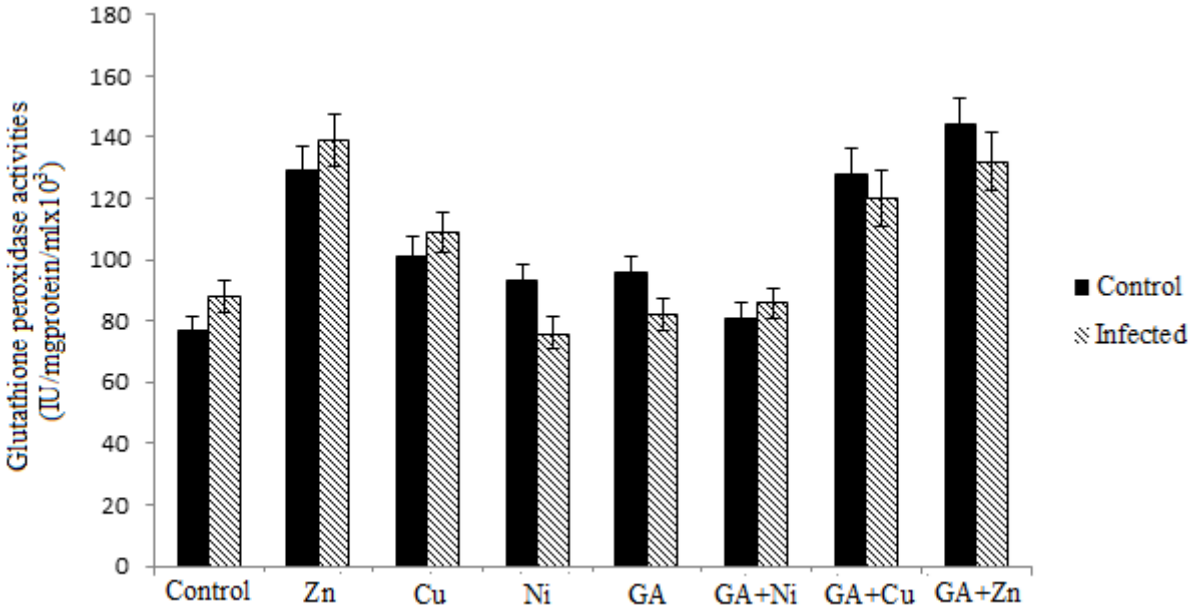

Figure 3

Comparison of glutathione peroxidase activities of Hyphantria cunea larvae in the control and the infected groups. Data are expressed as mean ± S.E. Two independent samples t-test, p <0.001