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Original article (Orijinal araştırma)

Determination of tannin activity and *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) on the biocontrol of tannin-tolerant *Agelastica alni* L., 1758 (Coleoptera: Chrysomelidae) larvae[1]

Tanen toleranslı *Agelastica alni* L., 1758 (Coleoptera: Chrysomelidae) larvalarının biyolojik mücadelesinde *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) ve tanen aktivitesinin belirlenmesi

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Abstract

Tannins are among the most abundant secondary metabolites synthesized by plants. *Agelastica alni* L., 1758 (Coleoptera: Chrysomelidae) is a critical forest pest. This study investigated the effect of *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) and tannins against *A. alni* larvae. The larvae were collected from the Çayeli district of Rize province in 2022. In the feeding experiments, artificial diets containing 1.25%, 2.5% and 5% tannins were prepared. 100 and 200 µl of *P. alvei* were applied to the infected groups. Nutritional indices, pupal masses, phenoloxidase activities, antioxidant enzyme activities and mortality rates of larvae fed with different diets were studied. Relative consumption rate (RCR) increased with tannin concentration in all groups. Relative growth rate (RGR) increased with rising tannin concentrations across all groups. In the infected groups, the increase in tannin concentration caused a decrease in developmental time. While superoxide dismutase and phenoloxidase activities of uninfected larvae decreased with tannin concentration, catalase and glutathione peroxidase activities of larvae increased. In infected larvae, catalase activity decreased with increasing tannin concentration. The dose of *P. alvei* caused an increase in superoxide dismutase and phenoloxidase activities, but did not affect catalase and glutathione peroxidase activities. The diet containing 5% tannic acid had the lowest mortality rate.

Keywords: *Agelastica alni*, antioxidant system, biocontrol, *Paenibacillus alvei*, tannin-tolerant

Öz

Tanenler, bitkiler tarafından sentezlenen en bol ikincil metabolitler arasındadır. *Agelastica alni* L., 1758 (Coleoptera: Chrysomelidae) önemli bir orman zararlısıdır. Bu çalışmada, *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) ve tanenlerin *A. alni* larvaları üzerindeki etkisi araştırılmıştır. Larvalar 2022 yılında Rize ili Çayeli ilçesinden toplanmıştır. Besleme deneylerinde, %1.25, %2.5 ve %5 tanen içeren yapay diyetler hazırlanmıştır. Enfekte gruplara 100 ve 200 µl *P. alvei* uygulanmıştır. Farklı diyetlerle beslenen larvaların beslenme indeksleri, pupal kütleleri, fenoloksidaz aktiviteleri, antioksidan enzim aktiviteleri ve ölüm oranları incelenmiştir. Nisbi tüketim oranı (RCR), tüm gruplarda tanen konsantrasyonu ile artmıştır. Nisbi büyüme oranı (RGR), tüm gruplarda tanen konsantrasyonunun artmasıyla yükselmiştir. Enfekte gruplarda tanen konsantrasyonundaki artış, gelişim süresinde azalmaya neden olmuştur. Enfekte olmayan larvalarda süperoksit dismutaz ve fenoloksidaz aktiviteleri tanen konsantrasyonu ile azalırken, katalaz ve glutatyon peroksidaz aktiviteleri artmıştır. Enfekte larvalarda ise tanen konsantrasyonundaki artış katalaz aktivitesinin azalmasına neden olmuştur. *P. alvei* dozu, süperoksit dismutaz ve fenoloksidaz aktivitelerinde artışa yol açarken, katalaz ve glutatyon peroksidaz aktivitelerini etkilememiştir. %5 tanik asit içeren diyet, en düşük ölüm oranına sahip olmuştur.

Anahtar sözcükler: *Agelastica alni*, antioksidan sistem, biyolojik mücadele, *Paenibacillus alvei*, tanen tolerant

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Introduction

Agelastica alni (L., 1758) (Coleoptera: Chrysomelidae), is an important pest of alder, hazel and willow trees. It has a wide distribution in Europe, the Caucasus, Siberia, north-eastern Kazakhstan and the USA. Its high reproductive rate causes defoliation of plants in natural habitats and parks, resulting in both economic and aesthetic damage. This condition also facilitates the invasion of host plants by other pests. The significant economic and aesthetic damage caused by *A. alni* is a cause for concern.

Chemical insecticides interfere with the physiological activities of target organisms. Pesticide residues also cause significant damage to environmental factors such as water and soil (Jayaraj et al., 2016). Due to the need for safer control methods, microbial insecticides, entomobacteria and their secondary metabolites are being used to control harmful insects (Yaman & Demirbağ, 2000; Sezen & Demirbağ, 2006; Isayama et al., 2021). *Paenibacillus* spp. Ash et al. (Bacillii: Paenibacillaceae) are widely distributed in the soil and rhizosphere (Atanasova-Pancevska & Kungulovski, 2018). Many *Paenibacillus* species can directly promote plant growth through biological nitrogen fixation, phosphate solubilization, production of the phytohormone indole-3-acetic acid (IAA), and release of siderophores that promote iron uptake. In this way, plants can protect themselves against herbivorous insects and phytopathogens, including bacteria, fungi, nematodes and viruses (Grady et al., 2016). It is also used as a biocontrol agent against many phytopathogenic fungi (Atanasova-Pancevska & Kungulovski, 2018). *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) is a secondary pathogen of American Foulbrood (AFB) in honeybees (Djukic et al., 2012). AFB is a fatal enteric disease. Spores germinate in the midgut lumen and vegetative bacteria multiply there before eventually attacking and penetrating the midgut epithelium, killing the larva (Poppinga & Genersch, 2015). It is effective in bees at low doses and lethal at high doses. However, there are few studies on the insecticidal activity of *Paenibacillus* species against other pests (Neung et al., 2014). Therefore, the insecticidal activity of *P. alvei* remains a gap in the literature.

Plants produce direct or indirect defense strategies against herbivore attack. Phytochemicals in direct defense strategies can influence herbivore preference and performance (Corry & Hoover, 2006). Tannins are one of the most abundant groups of phytochemicals in all vascular plants. They protect plants from insects by acting as deterrents or poisons. They are polyphenolic compounds of two types: hydrolysable tannins between 500-3000 Da and condensate tannins. Plant tannins act as protease inhibitors and can interact with digestive enzymes to precipitate proteins. This reduces protein utilization in herbivorous insects and impairs digestion (Pizzi et al., 2009). In the highly acidic environment of the insect gut, tannins undergo oxidation and bind to various enzymes, further disrupting digestive processes. They also contribute to food loss by binding to lipids and reducing their digestibility. Tannins also negatively affect insect development by inducing midgut lesions, which ultimately inhibit growth. They also act as a food deterrent due to their bitter taste (Price et al., 2019). In some plants, tannins also protect plants from harmful insects. Because of these properties, they can be used as an insecticide (Isayama et al., 2011, 2021; Mostafa et al., 2012; Djilali et al., 2021). In addition, studies show that the resistance of insects to viral and bacterial pathogens decreases with increasing tannin content in the leaves (Keating et al., 1989; Young et al., 1995; Lindroth et al., 1999). Host plants can modify the relationships between herbivorous insects and their pathogens. Changes in plant chemistry and structure can lead to differences in pathogen infection of insects. Hydrolysable tannins and condensate tannins have been shown to reduce viral mortality in Lymantria dispar and cotton bollworm (Corry & Hoover, 2006).

Various physiological responses, such as immune responses and intermediary metabolism, are present in insect haemolymph. Enzymatic changes in infected larvae would presume the metabolic stress experienced by insects during the development of the pathogen (Ibrahim et al., 2019). These enzymes control ROS (Reactive Oxygen Species) generated by biotic and abiotic stress in insects. ROS include free radicals, oxygen ions, and organic molecules. These radicals cause protein oxidation, lipid peroxidation,

nucleic acid damage, and activation of the immune system (Meşe et al., 2022). An increase in ROS elements is seen with exposure to pathogens and cell damage. Many studies have shown that the antioxidant system is a defense mechanism against pathogen-induced ROS production. The major antioxidant enzymes in insects are catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Vengateswari et al., 2020). SOD is the first detoxifying enzyme produced in cells and is a powerful antioxidant.

Insufficient degradation of superoxide anions or H_2O_2 can lead to the formation of hydroxyl radicals, which will cause the oxidation of sulfur residues in polypeptides or proteins and their degradation (Zhang & Feng, 2018). GPx is also essential in preventing lipid peroxidation and protecting cells from oxidative stress (Meşe et al., 2022). Another enzyme involved in humoral immunity is phenoloxidase. Phenoloxidase (PO) is a key enzyme that is activated by the prophenoloxidase (PPO) cascade in the cuticle or haemolymph of many insects as a defense response to immune challenges (Gillespie et al., 1997). During this process, the peptidoglycan recognition protein recognizes the appropriate elicitor and triggers the production of inactive prophenoloxidase. Phenoloxidase plays a crucial role in the conversion of phenols to quinones, which subsequently leads to melanin synthesis. Melanin is essential for defense against bacterial (Gram + and -) and fungal infections (Ibrahim et al., 2019).

In forest ecosystems, secondary metabolites can act against insect herbivores in two ways. They can either have a toxic effect on the larvae themselves or affect entomopathogens (Fernandez-Conradi et al., 2021). Therefore, the effects of both factors should be evaluated together to control forest pests. Plants of the genus *Alnus* are rich in tannins (Janceva et al., 2011). In the process of co-evolution, phytophagous insects have developed behavioral and physiological defense mechanisms to protect themselves from the harmful effects of secondary metabolites and to continue their population development under selective pressure. Behavioral mechanisms are designed to adapt feeding strategies and developmental rhythms. Physiological defense mechanisms are designed to reduce the toxicity of food and protect against oxidative damage (Jiang et al., 2021). *Agelastica alni* larvae feeding on alder leaves have also adapted to the tannins in the host plant during the coevolutionary process. The aim of our study is to investigate whether the tannins in *Alnus* leaves reduce their immune resistance when exposed to the entomopathogen *P. alvei*, even though they have adapted to feeding on host plants. In addition, this study aims to answer the question of whether *P. alvei* can be used as an entomopathogen to control other insect pests.

Materials and Methods

Obtaining larvae and preparation of artificial diets

The larvae were collected while feeding on alder leaves in the Çayeli district of Rize in 2022 and brought to the laboratory. They were fed on an artificial diet as a group in the laboratory. A total of four different artificial diets were used in the feeding experiment. The composition of the artificial diet, developed by Yamamoto (1969), is as follows: wheat germ, casein, sucrose, torula yeast, a vitamin mixture, cholesterol, sorbic acid, methyl paraben, linseed oil, agar, and distilled water. Diets containing tannic acid were prepared by adding tannic acid at concentrations of 1.25%, 2.5%, and 5%. The control diet did not contain tannic acid.

Preparing bacterial suspension

Paenibacillus alvei bacterial cultures were obtained from stock cultures at the Recep Tayyip Erdoğan University Microbiology Research Laboratory (deposited at DDBJ/ENA/GenBank under the accession number MZ673473). MYPGP (Mueller Hinton Broth, Yeast Extract, Potassium Phosphate, Glucose, Na-Pyruvate, Agar-agar) medium was used to prepare the cultures. An isolate of *Paenibacillus alvei,* collected from a deep freezer, was grown in MYPGP medium at 5% CO_2 and 36ºC temperature for 24-48 hours. The plate culture was inoculated as a single colony into 10 ml of MYPGP broth and incubated overnight at 36ºC in a shaking incubator at 120 rpm. Before use, the culture was diluted to 1×10^6 (cfu/ml) with sterile saline water.

Larval rearing

In the feeding trials, groups were established for all diets (no tannin, 1.25%, 2.5% and 5% TA) with no bacteria, and with 100 µl and 200 µl bacterial applications. A total of 12 experimental groups were set up. Larvae were placed in plastic petri dishes (10 cm x 3 cm) for each group. Fresh food was given every other day until the last larval stage, and the unconsumed food was replaced with fresh food. Bacteria were added using an automatic pipette inside a sterile cabinet. The procedure was repeated on each food replacement day.

Nutritional indices

When the larvae reached the last instar, as determined by the exuviae, 30 insects per experimental group were weighed and placed in plastic cups for individual feeding. The number of larvae used for the experiments was in accordance with the literature (Truzi et al., 2021). The last instar larvae were weighed on a balance with an accuracy of 0.001 mg at the beginning of the feeding, and their weight and the amount of food given were recorded. The diets were changed every two days, the remaining diets were packaged, and the weight gain of the larvae was recorded. The remaining diets were dried in an oven at 50ºC until they reached a constant weight. This process was continued until the larvae became pupae. Pupa mass was determined by drying the pupae at 50ºC until they reached a constant weight (Lee et al., 2002). Their developmental time was defined as the time required to reach the pupal stage.

According to Waldbauer (1968), consumption measured on the basis of the dry weight of the food reflects the rate at which nutrients enter the digestive system of an insect, whereas consumption calculated on the basis of the wet weight indicates the insect's behavioral response to the food. In this study, nutritional indices were assessed for the final instar larvae. The nutritional indices RCR and RGR were determined from fresh weight as described by Stochoff (1992). RGR is influenced by the quality of the host plant, the physiological condition of the insect and environmental factors. When calculations were based on dry weight, leaves, faeces and larvae were dried to a constant weight before measurement.

$$RCR\ (relative\ consumption\ sate) = \frac{dry\ weight\ of\ food\ consumed}{(insect\ weight\ at\ start\ of\ experiment)(time\ a)}$$

$$RGR\ (relative\ growth\ rate) = \frac{insect\ wet\ weight\ gain}{(insect\ wet\ weight\ at\ beginning\ of\ experiment)(time)}$$

Nutritional assays for enzyme activities

Larvae in the enzyme activity feeding groups continued to be fed together. As in the nutritional indices test group, the diets were changed every two days, and in the bacterial application groups, the application was made on the days when the nutrients were changed. Haemolymph samples for enzyme activities were collected two days before pupation. Hemolymph samples were collected from the larvae two days before pupation. For this purpose, the larvae were first sterilized with 95% ethanol. They were then collected by piercing the last proleg with a sterile needle. The haemolymph was extracted into Eppendorf tubes and stored at -27ºC until needed (Lee et al., 2008).

Haemolymph preparation for antioxidant enzyme activities

The haemolymph samples used in all enzyme activity assays were prepared as follows: 8 µL of hemolymph and 400 µL of ice-cold phosphate-buffered saline (PBS; pH 7.4) were mixed and vortexed. Samples were then frozen at -20ºC to disrupt haemocyte membranes (Wilson et al., 2001). The Bradford method was used to quantify the protein concentration of the haemolymph samples (Bradford, 1976).

Phenoloxidase activity assay

Phenoloxidase activity in haemolymph samples was assessed spectrophotometrically by following the formation of dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) from L-dihydroxyphenylalanine (L-DOPA). For this purpose, 100 µL of 10 mM L-DOPA (substrate) was combined with 100 µL of ice-cold phosphate-buffered saline (PBS, pH 7.4) haemolymph and incubated at 25ºC for 20 minutes (Wilson et al., 2001). After incubation, the absorbance of the mixture was measured at 492 nm.

Antioxidant enzyme activity assays

The superoxide dismutase activity of haemolymph samples was determined using a method based on the inhibition of nitroblue tetrazolium (NBT) reduction to formazan, which is then detected spectrophotometrically at a wavelength of 560 nm. A critical aspect of this method is the use of the xanthine-xanthine oxidase system as the superoxide generator. This system, a technique established by Beauchamp and Fridovich in 1971, is important because it provides a controlled and reliable source of superoxide ions for the assay. The reaction mixture, which was adjusted to a pH of 7.40 in a 10 mM phosphate buffer solution, contained 100 µM NBT and 50 µM xanthine. The concentration of xanthine oxidase in the reaction mixture was adjusted to produce an absorbance change of 0.025 min^{-1} at 560 nm.

According to Aebi (1984), the catalase activity of haemolymph samples was determined by measuring the decrease in H_2O_2 concentration spectrophotometrically at 240 nm. This reliable method involved mixing a freshly prepared hydrogen peroxide solution (0.036% (w/w). 2.80 mL of hydrogen peroxide solution in a quartz cuvette) with 20 µL of haemolymph samples and monitoring the change in absorbance (Shobha et al. 2016).

Glutathione peroxidase activity was measured spectrophotometrically using H_2O_2 as substrate according to Drotar et al (1985). The reaction mixture (250) contained 2 mM glutathione (GSH), 1 mM EDTA, 0.1 mM NADPH, 2.5 units of glutathione reductase, and 90 µM H_2O_2. Glutathione peroxidase activity was calculated from the rate of peroxide removal by following the change in absorbance at 340 nm resulting from the oxidation of NADPH in the reaction. The rate of NADPH oxidation was measured at 348 nm.

Determination of mortality rate

Confirmation of mortality rates in groups treated with different diets and different doses of *P. alvei* was performed using the Schneider-Orelli's formula (Hristov et al., 2022).

Statistical analyses

Statistical analyses of nutritional indices, developmental time, pupal mass, antioxidant enzyme activity, phenoloxidase activity and mortality rates were performed using SPSS version 23. Only the mortality rate follows a normal distribution. Two-Way ANOVA test was used to assess differences between groups. Tukey's Honestly Significant Difference (Tukey's HSD) test was used as a post hoc test to evaluate whether there were statistically significant changes. However, as the other parameters did not follow a normal distribution, the Kruskal-Wallis H test was used to assess group differences. Post hoc analysis was conducted with Dunn's test to observe statistically significant changes (p <0.05). Correlation tests were also performed to assess the relationships between the parameters.

Results

The effects of tannin concentrations and biocontrol agent application doses on the nutritional indices RCR and RGR, development time, and pupal mass were investigated. According to the results of the Kruskal-Wallis analysis, there are differences in the values of RCR, RGR, pupal mass, and development time according to the diets and *P. alvei* application dose (Table 1).

Table 1. Pairwise comparison results of nutritional indices, pupa mass, development duration indices, and antioxidant activities as a result of *Paenibacillus alvei* administration in different diets and at different doses

Parameters	N	X^2	df	*p*
RCR	180	168.37	11	0.00
RGR	180	158.56	11	0.00
Pupal Mass	180	163.99	11	0.00
Development Duration	180	173.88	11	0.00
Phenoloxidase Activity	180	165,07	11	0.00
SOD Activity	180	173,32	11	0.00
Catalase Activity	180	176,29	11	0.00
GPx Activity	180	177.09	11	0.00

Among all the diets, the highest RCR value was found in larvae fed on an uninfected 5% tannic acid diet. The lowest value was found in uninfected larvae fed on the control diet (no tannic acid) (Figure 1). When each tannic acid concentration was evaluated in isolation, infection had no significant effect except on the 2.5 % TA diet (Figure 1). As the tannin concentration increased in the uninfected groups, the RCR value also increased (r=0.29, $p<0.05$). In the infected groups, RCR increased with tannin concentration (r=0.92, $p<0.01$), but *P. alvei* dose had no effect on RCR ($p>0.05$). As the RCR increased in the non-infected groups, development time was prolonged (r=0.93, $p<0.01$) and pupal mass increased (r=780.52, $p<0.01$). Development time was shortened in the infected groups as RCR increased (r=-0.62, $p<0.01$).

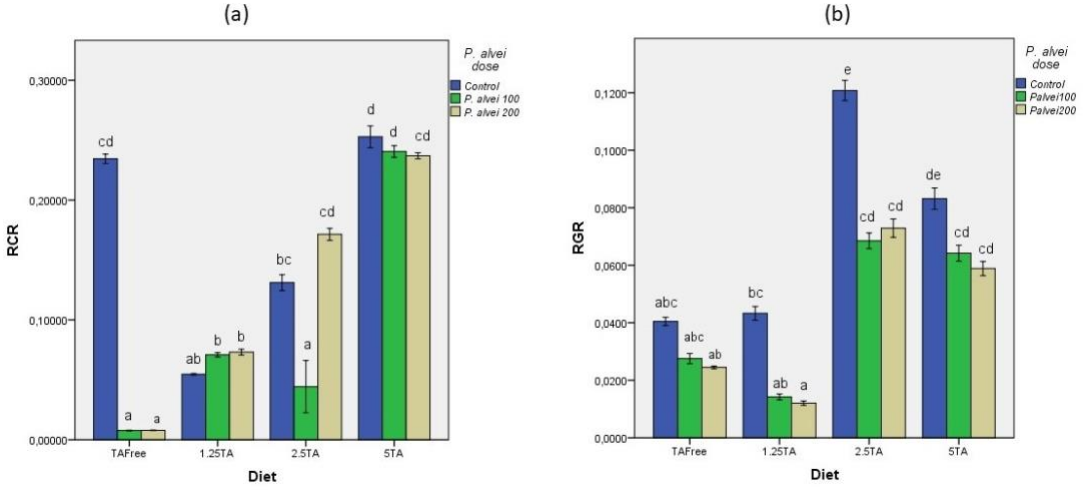

Figure 1. a) RCR values according to different tannin concentrations and different doses of *Paenibacillus alvei* applications (mean±standard deviation) b) RGR values according to different tannin concentrations and different doses of *Paenibacillus alvei* applications (mean±standard deviation).

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

In uninfected larvae, the highest RGR was observed in the 2.5 % TA diet (Figure 1). When the diet was examined separately at each tannic acid concentration, it was found that the RGR decreased with bacterial application (Figure 1). As the tannin concentration increased, the RGR increased in both the infected and uninfected groups (r=0.26, $p<0.01$; r=0.58, $p<0.01$). In the uninfected groups, as the RGR increased, the developmental period shortened (r=-0.29, $p<0.05$) and the pupal mass decreased (r=-0.47, $p<0.01$). In the infected groups, the pupal mass increased with increasing RGR (r=0.18, $p<0.05$).

The highest pupal mass value was recorded in the 5TA % diet, while the lowest pupal mass value was recorded in the group containing 200 µl of *P. alvei* with 1.25 % TA (Figure 2). The bacterial dose applied in the tannin-free diets had no effect on pupal mass (Figure 2). No relationship was found between tannin concentration and pupal mass in infected larvae ($p>0.05$). No correlation was found between pupal mass and *P. alvei* dose in infected groups ($p>0.05$). Pupal mass also increased with prolongation of development time in infected and non-infected groups (r=0.71, $p<0.01$; r=0.73, $p<0.01$).

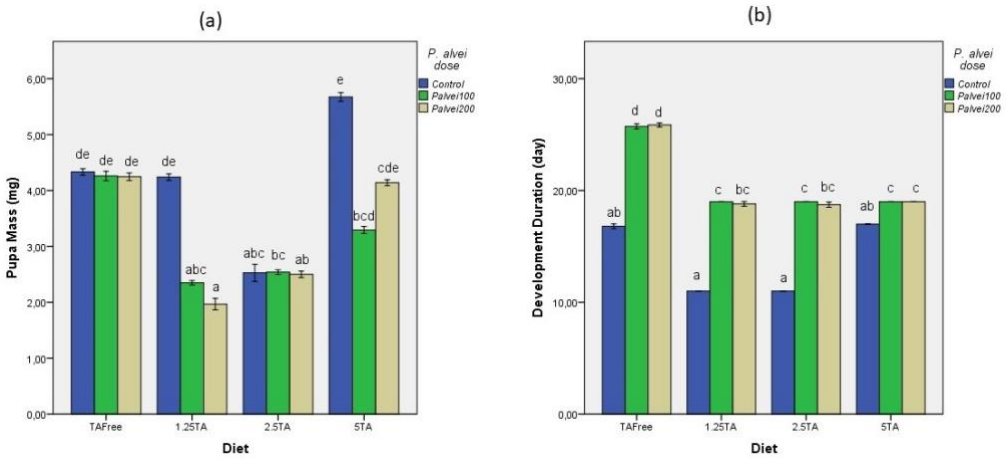

Figure 2. a) Pupal mass values (mean±standard deviation) according to different tannin concentrations and different doses of *Paenibacillus alvei* applications; b) Development times (mean±standard deviation) according to different doses of *Paenibacillus alvei* in diets containing different tannin concentrations.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

The longest development times were observed in tannic acid-free diets with 100 and 200 µl of *P. alvei.* The shortest development times were observed in 1.25 % TA and 2.5 % TA diets without *P. alvei* application (Figure 2). There was no correlation between tannin concentration and development time in the non-infected groups (p>0.05). In the infected groups, an increase in tannin concentration caused a decrease in the development time (r=-0.69, p<0.01). The application of *P. alvei* caused a prolonged development time (Figure 2). However, there was no correlation between dose and development time (p>0.05).

The enzyme activities of fhenoloxidase, catalase, SOD, and GSH-Px were examined. According to Kruskal-Wallis analysis, differences in enzyme activities were observed between groups (Table 1). The highest phenoloxidase activity was found when 200 µl of *P. alvei* was applied to the larvae fed the diet at 2.5 % TA concentration. The lowest activity was found in the larvae of the group treated with 100 µl of *P. alvei* on the 5 TA diet (Figure 3). Tannin concentration caused a decrease in phenoloxidase activity in the non-infected groups (r=-0.70, p<0.01). While the tannin concentration did not affect the infected groups (p>0.05), the dose of *P. alvei* caused an increase in phenoloxidase activity (r=0.76, p<0.01). In all the diets, especially the application of 200 µl in particular caused a significant increase in activity (Figure 3).

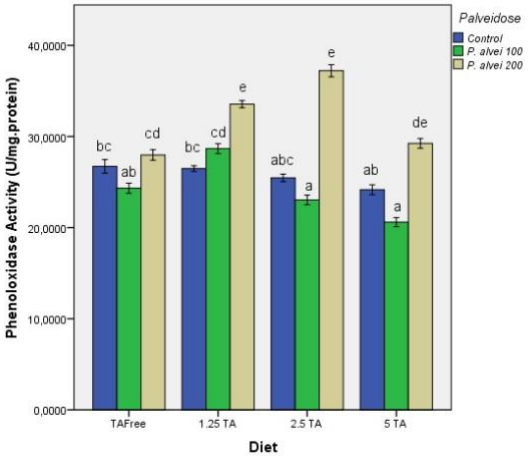

Figure 3. Phenoloxidase activities (mean±standard deviation) according to different tannin concentrations and different doses of*Paenibacillus alvei* applications.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

The highest SOD activity was detected in larvae fed on a 2.5% TA diet treated with 200 µl of *P. alvei*. The lowest SOD activity was found in the 5% TA diet without *P. alvei* application (Figure 4). SOD activity decreased with tannin concentration in the non-infected groups (r=-0.97, p<0.01). While tannin concentration did not affect SOD activity in infected groups (p>0.05), *P. alvei* dose caused an increase in activity (r=0.18, p<0.05). In contrast, this increase was seen in the tannin groups (Figure 4).

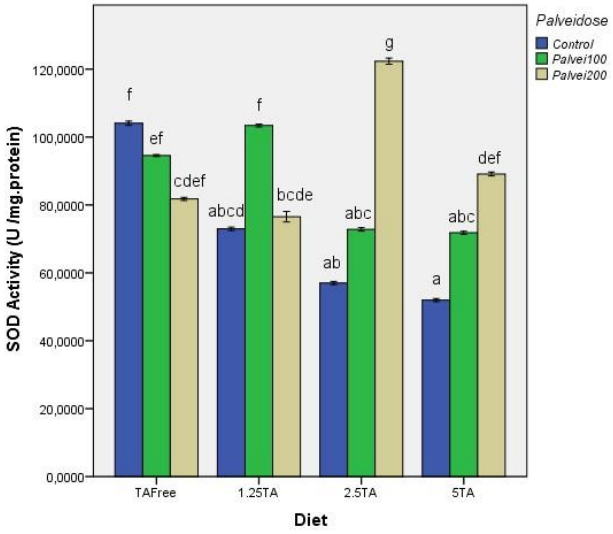

Figure 4. Superoxide dismutase activities (mean±standard deviation) according to different tannin concentrations and different doses of *Paenibacillus alvei* applications.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

The lowest catalase activity was found in the tannin-free diet treated with 100 µl of *P. alvei*. The highest activity was found in the larvae fed on the 1.25 TA diet treated with 100 µl of *P. alvei* (Figure 5). In the non-infected groups, enzyme activity increased with tannin concentration (r=0.74, p<0.01). In the infected groups, a decrease in activity with tannin concentration was observed (r=-0.26, p<0.01). However, the dose of *P. alvei* had no effect on catalase activity (p>0.05).

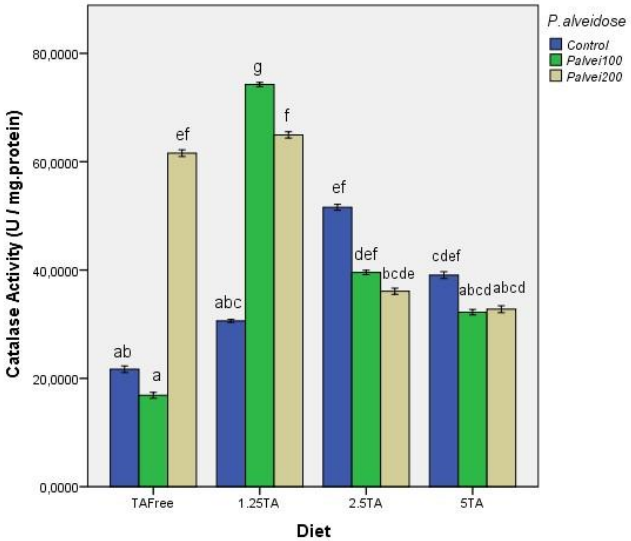

Figure 5. Catalase activities (mean±standard deviation) according to different tannin concentration and different dose *Paenibacillus alvei* applications.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

The lowest GPx activity was found in the diet containing no tannin to which 100 µl of *P. alvei* was applied. The highest activity was found in the diet containing 1.25% TA to which 100 µl of *P. alvei* was applied (Figure 6). Tannin concentration increased GSH-Px activity in the non-infected groups (r=0.77, p<0.01). In the infected groups, tannin concentration and *P. alvei* had no effect on GPx activity (p>0.05).

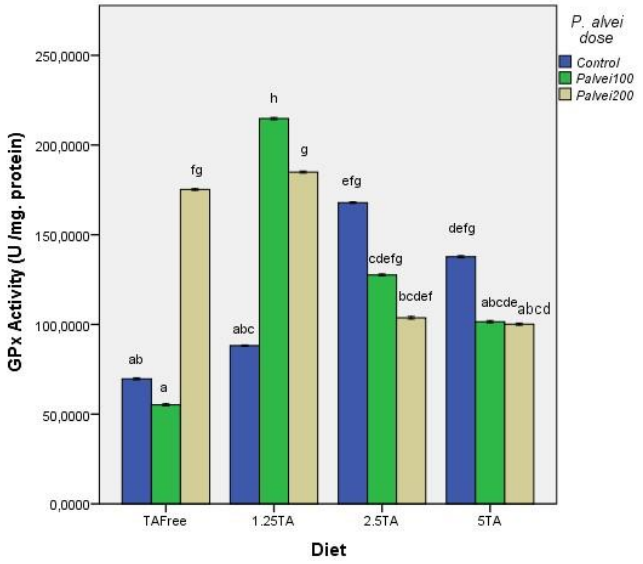

Figure 6. Glutathione peroxidase (mean±standard deviation) activities according to different tannin concentrations and different doses of *Paenibacillus alvei* applications.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

Correlation and regression analyses were performed to determine whether there was a relationship between RCR and RGR values and enzyme activity. The increase in the RCR in the uninfected groups caused only a decrease in phenoloxidase activity (r=-0.31, p<0.05). The increase in the RCR in the infected groups caused a decrease in catalase and GPx activities (r=-0.30, p<0.01; r=-0.31, p<0.01). In comparison, the increase in RGR in the uninfected groups caused an increase in catalase and GPx activities (respectively; r=0.95, p<0.01; r=0.97, p<0.01). It caused a decrease in SOD and phenoloxidase activities (respectively; r=-0.74, p<0.01; r=-0.4, p<0.01).

SOD activity increased with the increase in phenoloxidase activity (r=0.59, p<0.01), whereas CAT and GPx activity decreased (respectively; r=-0.42, p<0.01; r=-0.52, p<0.01) in the uninfected groups. While SOD activity increases, CAT and GPx activities decrease (respectively; r=-0.84, p<0.01; r=-0.86, p<0.01). Catalase and GPx activities are also directly positive (r=0.98, p<0.01). In the infected groups, while phenoloxidase activity increased, SOD, CAT, and GPx activities also increased (respectively; r=0.64, p<0.01; r=0.33, p<0.01; r=0.28, p<0.01). Catalase and GPx activities were positive (r=0.99, p<0.01).

Mortality rates have been corrected using the Schneider-Orelli formula. Mortality rates and standard errors are presented in Table 2. The highest mortality rate among the diets treated with *P. alvei* was observed in the larvae fed the diet without tannin and treated with 200 µl of *P. alvei*. The lowest mortality was observed in larvae fed on diets containing 5 % TA and diets treated with 100 µl of *P. alvei* (Table 2).

According to the results of the TUKEY test, the mortality rates of all diets were different (df11,24, F=302,797, p<0.01). As the data were normally distributed, the effect of *P. alvei* dose and dietary tannin concentration on mortality rate was determined by two-way ANOVA. While the effect of *P. alvei* alone was significant (F=14.73, p<0.01), no effect of tannin concentration alone on mortality was detected (p>0.05). However, the effect of *P. alvei* dose and tannin concentration on mortality was significant (F=168.22, p<0.01).

Table 2. Adjusted mortality rates using Schenider-Orelli's formula. No statistically significant difference is indicated by values with the same letter according to Tukey's HSD (0.05)

Diet	*Paenibacillus alvei* dose (µl)	Mortality (%)±Standard deviation
Control	-	12.37±0.12^{a}
	100	59.65±0.55^{b}
	200	85.73±1.29^{c}
1.25 TA	-	13.3±0.30^{d}
	100	57.05±1.03^{e}
	200	70.3±0.53^{f}
2.5 TA	-	40.63±0.60^{g}
	100	44.44±0.75^{h}
	200	58.96±0.49^{m}
5 TA	-	12.73±0.25^{n}
	100	42.99±0.37^{p}
	200	56.30±5.45^{r}

Discussion

Plants and pests are always in a relationship. In this relationship, plants develop physical and chemical defenses against herbivores. Secondary metabolites such as alkaloids, terpenoids, and tannins defend against pathogens and pests (Djilali et al., 2021). Tannins have larvicidal or deterrent properties in several insect orders, including Lepidoptera, Coleoptera, and Hemiptera (Hafeez et al., 2019; Tan et al., 2022). Tannins affect the development and fecundity of phytophagous insects. Ingestion through food or fumigation disrupts the integrity of the digestive system, resulting in death and malformation of the offspring (Djilali et al., 2021). In addition, phytophagous insects may reduce their food intake and activate their antioxidant and defense systems to protect their populations (Tan et al., 2022). The relative consumption rate (RCR) is often calculated as the weight of food consumed divided by the average body weight over the entire feeding period (Farrar et al., 1989). The highest RCR values of *A. alni* larvae were found in the larvae fed with 5% tannin and in the control group. The concentration of tannin in the larvae affected the RCR value of the larvae in the uninfected larvae. Similarly, the RGR values of the larvae increased with the tannin concentration. In herbivorous insects that cannot adapt to plant phenolics, toxic phenolics cause a decrease in RGR due to an inability to use the nutrient effectively, an inability to transform it, and intestinal paralysis (Deml et al., 1999). Although the RCR increased in the control diet containing no tannin, the RGR was low. However, in the diet containing 5% TA, the RGR and the RCR values are high. This result shows that the larvae are adapted to tannin and can use it. Larval mass and RGR values of gypsy moth larvae fed on tannin-containing oak and Robinia plants also decreased. Larvae use energy for development at the expense of the metabolic processes of tannins. Even if they are fed diets high in tannins for about 50 generations, their growth is negatively affected by tannins (Mrdakovic et al., 2013). The fact that the developmental time of larvae in the uninfected groups is not affected by the tannin concentration can be explained by adaptation.

There may be an antagonistic interaction between plant allelochemicals and the insecticidal activity of microorganisms. In this interaction, the feeding of herbivorous insects decreases, they ingest fewer microorganisms, and the insecticidal activity may decrease. Therefore, allelochemicals may reduce mortality. Conversely, allelochemicals may increase the insecticidal activity of microorganisms (Navon, 1992). In our study, in applications where tannin and *P. alvei* are present, RCR and RGR values increase with rising tannin concentrations and mortality decreases. Although similar to the results of Navon (1992) in terms of decreasing mortality and insecticidal activity of microorganisms, it differs in terms of decreasing consumption and RGR. The increase in the metabolic cost of immunity due to the application of *P. alvei* (Ardia et al., 2012) caused an increase in the protein and carbohydrate intake of the larvae. As a result, consumption and the RCR may have increased. As the larvae adapted to tannin, their convertibility increased and the RGR increased. The increase in pupal mass with the increase in RGR is also an indicator of usability. However, as pupal mass is an indicator of fecundity (Czypionka & Hill, 2007), the increased yield may suggest that biocontrol should not be applied during periods of high tannin concentrations.

When *P. larvae*, the causative agent of American foulbrood disease, enter the bodies of *Apis mellifera* L., 1758 (Hymenoptera: Apidae) larvae, they multiply in the midgut cells of the larvae and cause intestinal infection (Krongdan et al., 2019). During the invasive phase of the infection, the peritrophic matrix is degraded, and *P. larvae* penetrate the midgut epithelium. The bacteria enter the larval hemocoel, resulting in the larval death (Müller et al., 2015). *Paenibacillus alvei* is also a secondary pathogen of American Foulbrood. Therefore, *P. alvei* cause damage to the intestine. Krongdang et al. (2019) suggested that the reason why the disease is less common in *Apis cerana* Fabricius, 1793 (Hymenoptera: Apidae) may be more resistance due to the substances they ingest with their diet. Mortality of *A. alni* larvae is also lower in the % 5TA diet. Barbehenn and Martin (1992) found that the peritrophic membranes of tannin-tolerant *Orgyia leucostigma* Smith, 1797 (Lepidoptera: Erebidae) larvae were not permeable to high molecular weight tannins, and less than 1% of the grain in the diet was adsorbed. Again, Barbehenn et al (2001) found that the midgut of *O. leucostigma* larvae had higher concentrations of ascorbate and glutathione against oxidative stress than tannin-sensitive *Malacosoma disstria* Hübner, 1820 (Lepidoptera: Lasiocampidae) larvae. In addition, Summers and Felton (1996) stated that the protection of the midgut epithelium is a result of the antioxidant properties of the peritrophic membrane. *P. alvei* germinate and multiply in the midgut of larvae. In cases where tannin concentration is high, the excess of ascorbate and glutathione concentration in tannin-adapted larvae protects the midgut lumen from oxidative stress. This reduces the toxic effect of the microorganism. Therefore, mortality may be lower in 5TA diets.

Our study has opened up new avenues for further research in this area. Phenolic compounds can induce the antioxidant system. In our study, while tannin concentration caused a decrease in SOD and PO activities in the uninfected groups, an increase in CAT and GPx activities was observed. This result differs from the literature (Korayem et al., 2012; Tanet al., 2022). According to Korayem et al. (2012), *A. mellifera* increased SOD activity with phenolic compounds. Tannin stress inhibits antioxidant capacity in *Hyphantria cunea* Drury, 1793 (Lepidoptera: Erebidae) larvae. Catalase activity decreases with tannin stress (Tan et al., 2022). The presence of ROS and the deterioration of the antioxidant system indicate the presence of plant secondary metabolites with toxic effects. These findings not only contribute to our understanding of the effects of tannins and microorganisms on herbivorous insect larvae but also point to the need for further research in this area to fully understand the complex interactions involved.

Superoxide dismutase (SOD) is the first enzymatic defense against ROS. SOD enzymes convert negatively charged oxygen molecules to hydrogen peroxide, which is subsequently destroyed by catalase (Parker et al., 2004). Induction of SOD activity which rapidly destroys superoxide radicals appears to be the primary response to dietary pro-oxidant exposure (Ahmad & Pardini, 1990). Consumption of pro-oxidant compounds increases SOD and catalase activity. Tannins are also considered to be pro-oxidants. Tannin pro-oxidant activity and toxicity in herbivores is likely when tannins oxidise to form high levels of semiquinone radicals and quinones (Barbahenn & Constabel, 2011). In addition, the uptake of secondary metabolites is based on adaptation to a secondary metabolite involving antioxidant and detoxification enzymes (Tan et al., 2022). The decrease in SOD activity with tannin exposure in *A. aln*i larvae indicates that tannin is not a pro-oxidant for *A. alni* larvae and that they adapt to tannins. As catalase is one of the detoxification enzymes, the increase in catalase activity also shows the adaptation of larvae to secondary metabolites. An increase in tannin concentration and a decrease in phenoloxidase activity were observed in uninfected larvae. This result was reported by Sagona et al. (2021) and is in agreement with the present results. On the other hand, according to Sagona et al. (2021), tannin concentration decreased phenoloxidase activity in *A. mellifera*. Liu et al. (2010) found that tannin inhibited the phenoloxidase activity of *Spodoptera exigua* Hübner, 1808 (Lepidoptera: Noctuidae) larvae.

Enzyme activity increased with both presence and dose of *P. alvei* in infected groups. In addition, the mortality rate of larvae increased as the applied dose of *P. alvei* increased. The increase in enzyme activity also indicates that the immune system is stimulated. These results support the literature. Karthi et al. (2018) reported a significant increase in antioxidant enzymes of *Spodoptera litura* Fabricius, 1775 (Lepidoptera: Noctuidae) larvae infected with *Aspergillus flavus* Link (Eurotiales: Aspergillaceae).

Similarly, Ali et al. (2013) reported increased superoxide dismutase, catalase, and glutathione peroxidase activities in *S. exigua* larvae infected with *Isaria fumosorosea* Wize (Hyporeales: Cordycipitaceae). The antioxidant system is a defense mechanism against ROS production induced by pathogens and tannins. Various toxins released by microorganisms after bacterial or fungal infection cause activation of immune responses in insects or insect deaths (Lalitha et al., 2018). SOD is located in the mitochondrial intermembrane space and is the first enzyme to play a role in antioxidant defense. Baud et al. (2004) found that the high activity of catalase and low activity of GPx work in concert. However, catalase and GPx activities are directly proportional in both infected and uninfected groups of *A. alni* larvae. Jovanovic´-Galovic et al. (2004) found that catalase and GPx activities are directly proportional in *Ostrinia nubilalis* Hübner, 1796 (Lepidoptera: Pyralidae) larvae. Hydrogen peroxide is converted to water by either catalase or GPx enzymes (Baudet al., 2004). These enzymes are the cell's defense mechanism against hydrogen peroxide (Ali et al., 2013). Catalase is sensitive to high levels of H_2O_2, but GPx activity can also increase to maintain catalase activity. To overcome the cytotoxic effects of hydroxyl radicals and H_2O_2 under stressful growth conditions, both enzyme activities can be increased (Huarte-Bonnet et al., 2015).

Phenoloxidase is present in the haemolymph as an inactive zymogen. The increase in prophenoloxidase and phenoloxidase activity following the microbial or metazoan invasion is a stress response (Ashida & Brey, 1997). PO converts phenols to quinones and the subsequent production of melanin, which is essential for defense against bacterial (Gram + and −) and fungal infections (Eleftherianos & Revenis, 2011). In our study, the increase in *P. alvei* infection and the increase in phenoloxidase activity with its dosage is consistent with the literature.

In conclusion, the present study investigated the effect of *P. alvei* on the biocontrol of tannin-tolerant *A. alni* larvae. This research highlights the importance of considering secondary metabolites and tolerance to them in the biological control of pests, a crucial aspect that should be valued and respected in the fields of entomology, ecology and biological control. The results show that tannins, although generally considered toxic and repellent, can be tolerated or even utilized by adapted insect species such as *A. alni*, enhancing their growth and metabolic efficiency under certain conditions. Conversely, the entomopathogenic *P. alvei* significantly increases mortality and induces antioxidant and immune responses in larvae, the effects of which are modulated by the presence of tannins. The results suggest that the dual exposure to plant allelochemicals and microbial pathogens can have synergistic or antagonistic effects, depending on the physiological adaptations of the host insect. Furthermore, the observed changes in enzymatic activity highlight the importance of antioxidant and immune defenses in mediating insect responses to biotic stressors. These findings contribute to a better understanding of plant-insect-pathogen interactions and may inform future integrated pest management strategies, particularly regarding the timing and compatibility of botanical and microbial biocontrol agents.

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