Bulletin of Entomological Research

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Some *Trichoderma* strains and nitrogen fertilisation impact on *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae) larvae

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Research Paper

Cite this article: Topçu E, Altun N, Kılcı L, Karaoğlu ŞA, Faiz Ö (2025) Some *Trichoderma* strains and nitrogen fertilisation impact on *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae) larvae. *Bulletin of Entomological Research* **115**, 566–578. https://doi.org/10.1017/S0007485325100096

Received: 17 July 2024
Revised: 11 April 2025
Accepted 28 April 2025
First published online: 23 June 2025

Keywords:
biological control; *Cydalima perspectalis*; immunity; nitrogen fertilisation; nutritional indices; *Trichoderma harzianum*

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Abstract

Cydalima perspectalis (Walker, 1859) (Lepidoptera: Crambidae) larvae feed on *Buxus*. It is considered to be the most critical pest of boxwood trees. This study investigated whether different strains of *Trichoderma harzianum* had an effect on the biocontrol of larvae feeding on boxwood leaves whose nitrogen content was varied by fertilisation. Larvae were collected while feeding on boxwood seedlings in Rize parks and gardens in June 2021. In addition, G1 (no fertilisation), G2 (1.55%), and G5 (1.67%) leaves with different nitrogen concentrations obtained by nitrogen fertilisation were also used as food. As biocontrol agents, ID11D and YP1A strains of *T. harzianum* were applied in three doses: 50, 100, and 200 µL per water. In total, 21 different groups were created. The nutritional indices of the larvae belonging to the different groups were calculated. In addition, the activities of phenoloxidase, superoxide dismutase, catalase, and glutathione peroxidase activities were measured by taking haemolymph samples. In both strains, the enzyme activities increased with the dose applied. However, it was found that the enzyme activities of the ID11D strain applied were higher than those of the YP1A strain. It can be said that the ID11D strain is effective in controlling *C. perspectalis* larvae feeding on fertilised boxwood and the YP1A strain is effective in controlling larvae feeding on unfertilised boxwood.

Introduction

Boxwood is one of the most economically valuable timbers because it is heavy, fine-grained, and resistant to splitting and chipping (Bayramoğlu *et al.*, 2023). The species is slow growing and prefers moist and nutrient-rich soils (Bayramoğlu *et al.*, 2023). *Buxus sempervirens* is native to some parts of Europe, Africa, Asia, England, Morocco, Turkey, the Caucasus, and Iran (Chadburn and Barstow, 2018; Decocq *et al.*, 2004). Boxwood grows naturally in forests around the Black Sea, Thrace, the southern Marmara, and the eastern and western Mediterranean forests of Turkey (Lehtijarvi *et al.*, 2017). *Buxus sempervirens*, which generally spreads in the forest substrate, has established a natural population of approximately one thousand hectares in the forests of Turkey.

The primary hosts of the larvae of the invasive pest *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae) are boxwood species, including, *Buxus microphylla, Buxus microphylla var. insularis, B. sempervirens*, and *Buxus sinica*. The larvae feed on the leaves of boxwood, causing severe defoliation and tree mortality (Alkan Akıncı and Kurdoğlu, 2019). *C. perspectalis* is native to Europe (2007), southwestern Germany (Krüger, 2008), and Switzerland (Billen, 2007; Käppeli, 2008; Sigg, 2009), and is rapidly spreading to other European countries. The species, a very cosmopolitan pest, has been detected in Asia, America, and Africa (Can *et al.*, 2022). It was first detected in Turkey in 2011 in parks and gardens in the Sarıyer district of Istanbul (Hızal *et al.*, 2012). It was detected in Düzce, Artvin, Bartın, and Kastamonu (Öztürk *et al.*, 2016; Yıldız *et al.*, 2018), in Rize Kaçkar Mountains National Park in 2019 (Toper Kaygın and Taşdeler, 2019), and in Hatay in 2022 (Ak *et al.*, 2021).

The effectiveness of different microorganisms as biocontrol applications against *C. perspectalis* has been tested. *Beauveria bassiana* (Dong *et al.*, 2022; Zamani *et al.*, 2018), two isolates of *Anagrapha falcifera* nucleopolyhedrovirus named Dn10 and BI-235 (Rose *et al.*, 2013), *Isaria fumosorosea* (Zemek *et al.*, 2020) and the activities of *Bacillus thuringiensis* (Berliner) have been studied (Las Heras and Arimany, 2020; Las Heras *et al.*, 2019). Rose *et al.* (2013) examined that the virulence of the various isolates of *A. falcifera*, demonstrating the BI-235 isolate's LC50 value was 7.8 × 10(5) OBs/mL, and the Dn10 isolate's LC50 value was 2.3 × 10(6) OBs/mL. Zemek *et al.* (2020) applied 1×10^4 to 1×10^8 spores per 1 mL to determine the effectiveness of the CCM

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8367 strain of *I. fumosorosea* on the last larval stage of *C. perspectalis*. It was reported that the effectiveness of the applied doses and strain on pupal mortality was at most 60%. There are also studies on the effectiveness of entomopathogenic nematodes (Choo *et al.*, 1991; Yaman, 2023). Entomopathogenic nematodes are obligate parasites and reproduce in insect larvae (Van der Linden *et al.*, 2022). *Steinernema carpocapsae* caused 97.8–100% mortality in *C. perspectalis* larvae under laboratory conditions (Choo *et al.*, 1991). Fungi belonging to the *Trichoderma* genus can cause adverse effects on the roots of plants (Contreras-Cornejo *et al.*, 2016). However, in recent years, there has been evidence that *Trichoderma* supports plant growth and has a biocontrol effect against plant pathogens (Poveda, 2021). *Trichoderma harzianum* strain 101645 and strain 206040 have shown insecticidal activity when ingested by *Tenebrio molitor* larvae or when applied to the cuticle with serine protease (Shakeri and Foster, 2007).

Insects encounter entomopathogens, metabolic processes, and immune responses within the insect body (Jiang *et al.*, 2020; Qu and Wang, 2018). Phenoloxidase (PO), one of the humoral immune enzymes, is a critical defense factor against pathogens or insecticides (Jiang *et al.*, 2020). PO activation causes the formation of a melanin coating around the pathogen and chemically involves the formation of short-lived reactive quinones (Lalitha *et al.*, 2018). Another reaction that occurs when pathogens are encountered is the generation of reactive oxygen species (ROS). These free radicals cause protein oxidation, lipid peroxidation, nucleic acid damage, and activation of the immune system (Meşe *et al.*, 2022). Insects also have various antioxidant and detoxification enzymes that scavenge free radicals and function in the biotransformation of toxic metabolites. The most important of these enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Lalitha *et al.*, 2018). SOD is the first line of defense against ROS. SOD scavenges superoxide anions and detoxifies them by converting them into hydrogen peroxide and oxygen. Hydrogen peroxide is then converted into water and oxygen by CAT and GPx (Lalitha *et al.*, 2018).

Herbivore insects obtain all the nutrients necessary for their development from the plants they feed on. Nitrogen, one of the nutrients, is found in the protein and DNA structure and affects fecundity. In addition, the amount of nitrogen in the plant affects the development rates of pests, development times, larval period length, life span, and population density (Bala et al., 2018). The world is experiencing dramatic climate changed and increased denitrification caused by global warming reduces the availability of nitrogen in the soil. The increase in nitrogen has critical consequences, especially in nitrogen-poor soils. The lost nitrogen having to be replaced (Dong *et al.*, 2022). Nitrogen fertilisation stimulates plant grow and development by providing the necessary nutrients (Singh and Sood, 2017). Appropriate application changes the quality of plant nutrients and defence mechanisms, directly affecting insects that feed on plants (Chen *et al.*, 2008). Insect feeding includes food intake, absorption, assimilation, biosynthesis, catabolism, and excretion (Bala et al., 2018). Calculation of nutritional indices using the gravimetric method developed by Waldbauer (1968) has been used for many years to understand changes in consumption and assimilation (Stoyenoff *et al.*, 1994), determine insect performance on different host plants (Mehrkhou *et al.*, 2013), measure insects physiological stress responses (Nathan *et al.*, 2005; Rayapuram and Baldwin, 2006), and determine host plant resistance (Nathan, 2013). Essential nutritional indices are approximate digestibility (AD) of food, efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), relative consumption rate (RCR), and relative growth rate (RGR) (McEwan *et al.*, 2009).

In the study, it was investigated whether nitrogen fertilisation has an effect on the biocontrol of *C. perspectalis* larvae inoculated with different strains of *T. harzianum*. Boxwood seedlings are fertilised because they like nutrient-rich soils and are used for landscaping purposes in parks, gardens, and forestry. Therefore, it was aimed to carry out more effective biocontrol by determining whether the effects of the strains were different in the areas where fertilisation was done. Two different strains of *T. harzianum* were used in the study. The YP1a strain was isolated from garden soil in the Kirazlık neighbourhood of Pazar district, Rize province, in August 2014. YP1a was identified as a highly antagonistic strain against *Fusarium* species (12 different strains), *Sclerotium sclerotia* and *Botrytis cinerea*, which are considered responsible for diseases such as wilting and damping-off in tomato and pepper plants. According to Kasap (2018), YP1a showed higher efficacy in tomato seed germination (70% success rate) compared to the control, and significantly improved root, stem, and leaf development, as well as water-holding capacity in tomato plants. The ID11D strain, on the other hand, was isolated from the soil of tea plantations in İkizdere Valley, Rize, as reported by Alpay Karaoglu and Ülker (2006). It is preserved in the culture collection of Recep Tayyip Erdoğan University Biology Department as a *Trichoderma* spp. strain from İkizdere (ID) tea soil. Bozdeveci (2014) found that the ID11D strain exhibited high antifungal and antibacterial activity. Bozdeveci (2014) also found that ID11D has high chitinase activity. The aim of this study was to investigate the effects of these two different strains in pest control. For this purpose, nutritional indices of larvae fed on leaves with different nitrogen amounts and treated strains were calculated. Nutritional indices are a factor that determines whether the larvae use the consumed plants, as even if the plant is consumed, the larvae may not use it for development. Knowing this will also be helpful for the control. In addition to nutritional indices, physiological changes in larval immune, and antioxidant systems due to fertilisation and infection with different *T. harzianum* strains were examined.

Material and methods

Larval and plant sampling

C. perspectalis larvae were collected while feeding on boxwood (*Buxus microphylla*) seedlings in the parks and gardens of Rize province in June 2021. The collected larvae were brought to the laboratory and placed in transparent containers. A sufficient number of *B. microphylla* leaves to be used in feeding experiments were collected from the same region at the same time as the larvae and stored at +4°C to ensure that the amount of secondary metabolites and nitrogen did not change with plant growth. Plant samples that had not previously been treated with insecticide or fertiliser were selected.

Fungal culture and preparation of spore suspension

Two strains of *T. harzianum*, ID11D and YP1A, were used to infect to plant samples. Strain YP1A was isolated from a tomato garden in Pazar district, Rize province (Kasap, 2018), and the strain ID11D was isolated from the İkizdere district, Rize province (Bozdeveci, 2014). Potato dextrose agar was used for fungal culture. According

https://doi.org/10.1017/S0007485325100096 Published online by Cambridge University Press

to the manufacturer's recommendation, the medium was weighed at 39 g/L and sterilised in an autoclave at 121°C for 20 min. After allowing it to cool slightly, it was poured into sterile Petri dishes and used after cooling and freezing. The *Trichoderma* isolates were revived from glycerol stock culture at −80° for use in experiments and used in the study. For the spore suspensions to be used in the study, fungal cultures were incubated at 28°C for 7–10 days after single colony inoculation on Sabouraud dekstrose agar media. The same method was used for all fungal suspensions prepared throughout the study. A spore suspension containing 1×10^7 cfu/mL spores from a 7- to 10-day culture was used in this study.

Nitrogen fertilisation and feeding experiment

Leaves with three different levels of nitrogen were used in the study. Nitrogen fertilisation was applied to achieve the desired nitrogen level. There was also a group with no fertilisation. NCS fertiliser was used in the laboratory at concentrations of 2.5% and 5%. The leaves to be used for fertilisation were kept in an aqueous solution prepared at 2.5% (G2) and 5% (G5) concentrations for 12 h and then used in feeding experiments (Firidin and Mutlu 2009). In the study, leaves without fertilisation were referred to as G1. As the study aimed to investigate the effects of two different cultivars, separate experimental groups were established for each cultivar. In the feeding trials, where nutritional index and pupal mass were determined, four different feeding groups were established for each nitrogen concentration. No fungus was applied to one group. The other groups were treated with 50, 100, and 200 µL doses of the YP1A strain. The same feeding groups were prepared for the ID11D strain and the same doses were applied. This resulted in a total of 21 different experimental groups. The names of the feeding groups are given in table 1.

In the feeding experiments, 15 larvae were individually placed in each group to determine their nutritional indices. The last larval stage was used to calculate the nutritional indices. Food was given to the larvae at 1-day intervals, and at each change of food, the weight of the larvae was measured and recorded on a precision balance to an accuracy of 0.0001 units. The fungal applications were also repeated on the days when the diet was changed. Feeding and weighing continued until the larvae pupated. The remaining food was labelled and dried in an oven at 50°C until it reached a constant weight. The consumption of the larvae was then calculated. The faeces of the last larval stage were collected and dried to be used in the calculation of nutritional indices. Pupae were kept in an oven at 50°C until they reached a constant weight, and pupal mass was calculated.

Nutritional indices for all experimental groups were calculated for the last larval instar as formulated by Stoyenoff *et al.* (1994) (table 2). Nutritional indices are AD, convertibility of digested food (ECD), convertibility of ingested food (ECI), RGR ratio, and RCR.

A second feeding group was established to determine enzyme activities. Each feeding group was also set up for these experiments. However, the larvae in each experimental group were fed collectively rather than individually. Fungal applications and food changes were repeated every other day. In these feeding groups, pre-pupa larvae were first sterilised with 95% ethanol, the last proleg was punctured with a sterile needle, and the haemolymph was collected in Eppendorf tubes. It was frozen at −27°C until needed (Lee *et al.*, 2008). The collected haemolymph samples were used for biochemical analyses.

Table 1. Feeding groups set up with larvae fed on leaves containing different concentrations of nitrogen and treated with different doses of different trichoderma strains

Diet groups	Nitrogen amount of diets	Applied strain/dose (µL)
G1	Not fertilized	Not applicable
YP1A50	Not fertilized	YP1A/50
YP1A100	Not fertilised	YP1A/100
YP1A200	Not fertilised	YP1A/200
ID11D50	Not fertilised	ID11D/50
ID11D100	Not fertilised	ID11D/100
ID11D200	Not fertilised	ID11D/200
G2	2.5% Nitrogen fertiliser	Not applicable
G2YP1A50	2.5% Nitrogen fertiliser	YP1A/50
G2YP1A100	2.5% Nitrogen fertiliser	YP1A/100
G2YP1A200	2.5% Nitrogen fertiliser	YP1A/200
G2ID11D50	2.5% Nitrogen fertiliser	ID11D/50
G2ID11D100	2.5% Nitrogen fertiliser	ID11D/100
G2ID11D200	2.5% Nitrogen fertiliser	ID11D/200
G5	5% Nitrogen fertiliser	Not applicable
G5YP1A50	5% Nitrogen fertiliser	YP1A/50
G5YP1A100	5% Nitrogen fertiliser	YP1A/100
G5YP1A200	5% Nitrogen fertiliser	YP1A/200
G5ID11D50	5% Nitrogen fertiliser	ID11D/50
G5ID11D100	5% Nitrogen fertiliser	ID11D/100
G5ID11D200	5% Nitrogen fertiliser	ID11D/200

Preparing haemolymph for enzyme activities

Haemolymph samples were prepared for all enzyme activity assays as follows. And 8 µL haemolymph and 400 µL ice-cold phosphate-buffered saline (pH 7.4) were mixed and vortexed. Samples were frozen at −20°C to disrupt haemocyte membranes (Wilson et al., 2001). The total protein concentration of the haemolymph was measured according to the Bradford method (Bradford, 1976).

PO activity measurement

PO activity was determined according to the protocol of Lee *et al.* (2008). The buffered haemolymph solution prepared in the previous step was transferred to another Eppendorf tube, and 100 µL of 10 mM L-dopa (substrate) solution was added. The mixture was incubated at 25°C for 20 min. At the end of the incubation period, the absorbance of the mixture was measured at a wavelength of 492 nm using a microplate reader. Enzyme activity was expressed as U/mg protein.

Antioxidant enzyme activity

Superoxide dismutase activity in haemolymph samples was determined spectrophotometrically by a method that prevents the reduction of nitroblue tetrazolium (NBT) to formazan (Beauchamp and Fridovich, 1971). The reaction mixture was

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Table 2. Nutritional indices formula (Stoyenoff et al., 1994)

Nutritional indices	Formula
Approximate digestibility (AD)	$\left(\frac{Dry\ weighed\ of\ food\ consumed - the\ dry\ weighed\ of\ feces}{dry\ weighed\ of\ food\ consumed}\right) \times 100$
Efficiency of conversion of digested food (ECD)	$\left[\frac{insect\ dry\ wt\ .gain}{dry\ wt\ .\ food\ eaten - dry\ wt\ .\ frass}\right] \times 100$
Efficiency of conversion of ingested food (ECI)	$\left[\frac{insect\ dry\ wt\ .\ gain}{dry\ wt\ .\ food\ eaten}\right] \times 100$
Relative concumption rate (RCR)	$\frac{dry\ wt\ .\ food\ eaten}{insect\ wet\ wt\ .\ at\ the\ beginning\ of\ the\ trial}\ time$
Relative growth rate (RGR)	$\frac{insect\ wet\ wt\ .\ gain}{insect\ wet\ wt\ .\ at\ the\ beginning\ of\ the\ trial}\ time$

carried out in a medium containing 100 µM NBT and 50 µM xanthine in 10 mM phosphate buffer solution (pH 7.40). The concentration of xanthine oxidase was adjusted to give a change in absorbance of 0.025/min at a wavelength of 560 nm in the reaction mixture (final concentration of 0.01 U/mL). Enzyme activity was expressed as U/mg protein.

CAT activity was determined spectrophotometrically by measuring the decrease in hydrogen peroxide (H_2O_2) concentration at 240 nm (Aebi, 1984). A freshly prepared hydrogen peroxide solution with a concentration of 0.036% (w/w) was used. The change in absorbance was monitored by mixing 2.80 mL of hydrogen peroxide solution with 20 µL of haemolymph sample in a quartz cuvette. Enzyme activity was expressed as U/mg protein.

GPx activity was measured spectrophotometrically according to the method developed by Drotar *et al.* (1985). H_2O_2 was used as the substrate. The reaction mixture (250 µL) contained 2 mM glutathione (GSH), 1 mM EDTA, 0.1 mM NADPH, 2.5 units of GSH reductase, and 90 µM HO. GPx activity was calculated from the rate of peroxide removal rate determined by monitoring changes in absorbance at 340 nm resulting from oxidation of NADPH. The rate of oxidation of NADPH was measured at 348 nm. Enzyme activity was expressed as U/mg protein.

Mortality rate

The mortality rate for each group was corrected using the Sun–Shepard formula according to Püntener (1981) (Atwa, 2018; Emilie *et al.*, 2015).

$$Corrected\ (\%) = \left(Mortality\ \%TP \pm change\ \%in\ CPP \pm \frac{change\%CPP}{100}\right) x\ 100$$

$$Change\ \%in\ CP\ mortality = \left(\frac{CPP\ afterT - CPP\ before\ T}{CP\ before\ T}\right) X\ 100$$

where TP = treated plot; CPP = control plot population, in this study control treatment; and T = treatment.

Statistical analyses

AD values of larvae treated with the YP1A strain and mortality rates of larvae treated with both strains were normally distributed (Kolmogorov–Smirnov normality test), analysis of variance (ANOVA) was used, followed by Tukey's Honestly Significant Difference test was used to compare the effect of different strains on larval parameters. The data for the other parameters are non-parametric. Therefore, the difference between the groups was determined by a Kruskal–Wallis test with pairwise comparison. A two-way ANOVA test was used to determine the effect of *T. harzianum* strain dosage and nitrogen amount on *C. perspectalis* survival, nutritional indices, enzyme activities, and mortality rate. For this purpose, square root transformation was used to bring non-parametric groups closer to a normal distribution. A correlation test was performed to determine the relationship of the parameters with dose and nitrogen level, followed by multiple regression analysis. SPSS version 23.0 was used for statistical analysis.

Table 3. Nitrogen amounts in leaves ± standard error

Diet	Nitrogen amounts (%) ± Standard error
G1	1.40 ± 0.01[a]
G2	1.55 ± 0.02[b]
G5	1.67 ± 0.02[c]

Note: Different letters indicate differences between groups ($df = 2$, $F = ı81.63$, $p < 0.01$).

Results

In the study, the nitrogen content of the leaves was changed by fertilisation using NPC fertiliser. Nitrogen amounts of leaves used in feeding experiments are given in table 3. However, the amounts of phosphorus and carbon did not differ significantly between the groups ($p > 0.05$).

Nutritional indices and pupal mass

This study investigated the effects of different *T. harzianum* strains on the nutritional indices and mortality of larvae fed on plants with different levels of nitrogen. The highest AD value of larvae treated with the strain YP1A was found in the YP1A200 diet, and the lowest value was found in the uninfected larvae fed on the G2 diet. However, the lowest AD value in the group treated with the ID11D strain was in the larvae fed on the G5 diet and treated with 100 µL of fungal suspension and the G1 diet treated with 50 µL of fungal suspension. In larvae treated with both strains, the amounts of nitrogen and dosage affected the AD value (two-way ANOVA, YP1A; $df = 6$, $F = 3.93$, $p < 0.01$; ID11D; $df = 6$, $F = 984.93$, $p < 0.01$). However, while the AD value of larvae treated with the strain YP1A increased with the dose, it decreased with the amount of nitrogen. However, no correlation was found between nitrogen level, dose, or AD value for strain ID11D (table 4).

The lowest ECD value was found in larvae fed on the G1 diet for both strains. In contrast, the highest ECD value in larvae treated with strain YP1A was recorded in larvae fed on the G2 diet and treated with 200 µL of fungal suspension. The highest value for the strain ID11D was determined in the larvae fed on the G5

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Table 4. Correlation coefficients between nutritional indices and enzyme activities of larvae and nitrogen content of the plant and application dose of the treated strain

			Strain dosage	Nitrogen
AD	YP1A	*r*	0.29	−0.33
		p	<0.01	<0.01
	ID11D	*r*	–	–
		P	>0.05	>0.05
ECD	YP1A	*r*		−0.17
		p	>0.05	<0.05
	ID11D	*r*	0.20	0.49
		p	<0.01	<0.01
ECI	YP1A	*r*	–	−0.17
		p	>0.05	<0.05
	ID11D	*r*	0.21	0.52
		p	<0.01	<0.01
RCR	YP1A	*r*	0.43	−0.47
		p	<0.01	<0.01
	ID11D	*r*	−0.18	–
		p	<0.05	>0.05
RGR	YP1A	*r*	0.22	–
		p	<0.01	>0.05
	ID11D	*r*	−0.17	–
		p	<0.05	0.05
Pupa mass	YP1A	*r*	–	−0.19
		p	>0.05	<0.05
	ID11D	*r*	0.18	–
		p	<0.01	>0.05
Phenoloxidase	YP1A	*r*	0.64	−0.3
		p	<0.01	<0.01
	ID11D	*r*	0.83	–
		p	<0.01	>0.05
Superoxide dismutase	YP1A	*r*	–	−0.4
		p	>0.05	<0.01
	ID11D	*r*	0.61	0.45
		p	<0.01	<0.01
Catalase	YP1A	*r*	–	–
		p	>0.05	>0.05
	ID11D	*r*	–	–
		p	>0.05	>0.05

(*Continued*)

diet and treated with 100 µL of fungal suspension (fig. 1). In larvae treated with both strains, the amount of nitrogen and the dose together influenced the ECD value (two-way ANOVA; YP1A, $df = 6, F = 1271.34, p < 0.01$; ID11D, $df = 6, F = 275.53, p < 0.01$). However, in the strain YP1A, all parameters individually had no effect. As indicated by the higher *F* value, the YP1A strain has a greater effect on the ECD values in comparison to the ID11D strain. The ECD value for YP1A decreases as the amount of nitrogen increases, although no relationship could be established. In the strain ID11D, the ECD value increased with both the

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Table 4. (*Continued.*)

			Strain dosage	Nitrogen
Glutathione peroxidase	YP1A	*r*	0.79	0.18
		p	<0.01	<0.05
	ID11D	*r*	0.55	−0.5
		p	<0.01	<0.01
Mortality rate	YP1A	*r*	0.45	−0.48
		p	<0.01	<0.01
	ID11D	*r*	0.58	–
		p	<0.01	>0.05

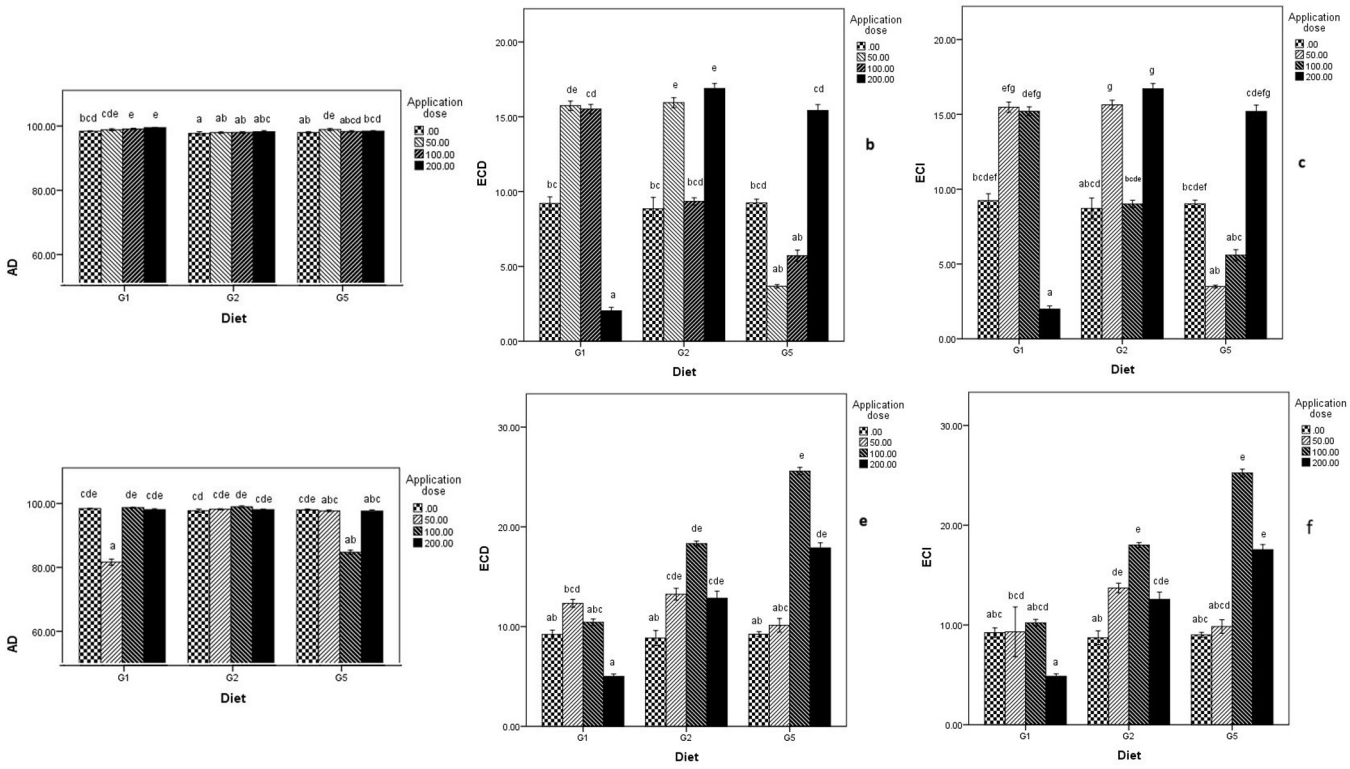


Figure 1. AD, ECD, and ECI of *C. perpectalis* larvae treated with different strains *trichoderma* (a–c) YP1A strain; (d–f) ID11D strain).
Note: *Nutritional indices differ among the groups. For AD: YP1A; $F = 14.78$, $df = 11$, $p < 0.01$; for ID11D, $F = 11{,}375$, $df = 11$, $p < 0.01$. ECD: YP1A; $X^2 = 163.84$, $df = 11$, $p < 0.01$; ID11D: $X^2 = 165.6$, $df = 11$, $p < 0.01$. ECI: YP1A; $X^2 = 164.27$, $df = 11$, $p < 0.01$; ID11D; $X^2 = 153.91$, $df = 11$, $p < 0.01$.

amount of nitrogen ($r = 0.49$, $p < 0.01$) and the application dose (table 4).

The lowest ECI values for both strains were in the G1 diet without fertlisation, was 200 µL of fungal suspension was applied. The highest value was found for larvae fed on the G5 diet and treated with 100 µL of YP1A fungal suspension. For the strain ID11D, the highest value belongs to the larvae fed on the G5 diet and treated with 100 µL of fungal suspension (fig. 1). The amount of nitrogen and dosage affected the ECI value ($F = 1319.39$, $p < 0.01$) for strain YP1A. The ECI decreased as the amount of nitrogen increased. No relationship was found between application dosage and ECI value (table 4). For the ID11D strain, the amount of nitrogen and the application dosage influenced the ECI value (two-way ANOVA, $df = 6$, $F = 44.51$, $p < 0.01$). The ECI value was found with increasing nitrogen amount and application dose (table 4).

The lowest RCR value was found in the larvae fed on the G5 diet and treated with 50 µL of fungal suspension for the strain YP1A. For strain ID11D, the lowest value belongs to the larvae fed on the G5 diet and treated with 50 µL of fungal suspension (fig. 2). The highest RCR value was obtained with the YP1A200 diet for strain YP1A. For ID11D strains, RCR values were higher for uninfected larvae fed on G1 and G5 diets (fig. 2). In contrast to the AD and ECD values, the highest RCR values were found for the YP1A strain. In larvae treated with both strains, YP1A and ID11D, the amount of nitrogen and the application dose together affected the RCR value (respectively; $df = 6$, $F = 117.87$, $p < 0.01$; $df = 6$, $F = 386.07$, $p < 0.01$). The RCR value decreased as the amount of nitrogen increased, and the value increased as the applied dose increased for the strain YP1A strain. However, the amount of nitrogen does not affect the RCR value for the ID11D strain.

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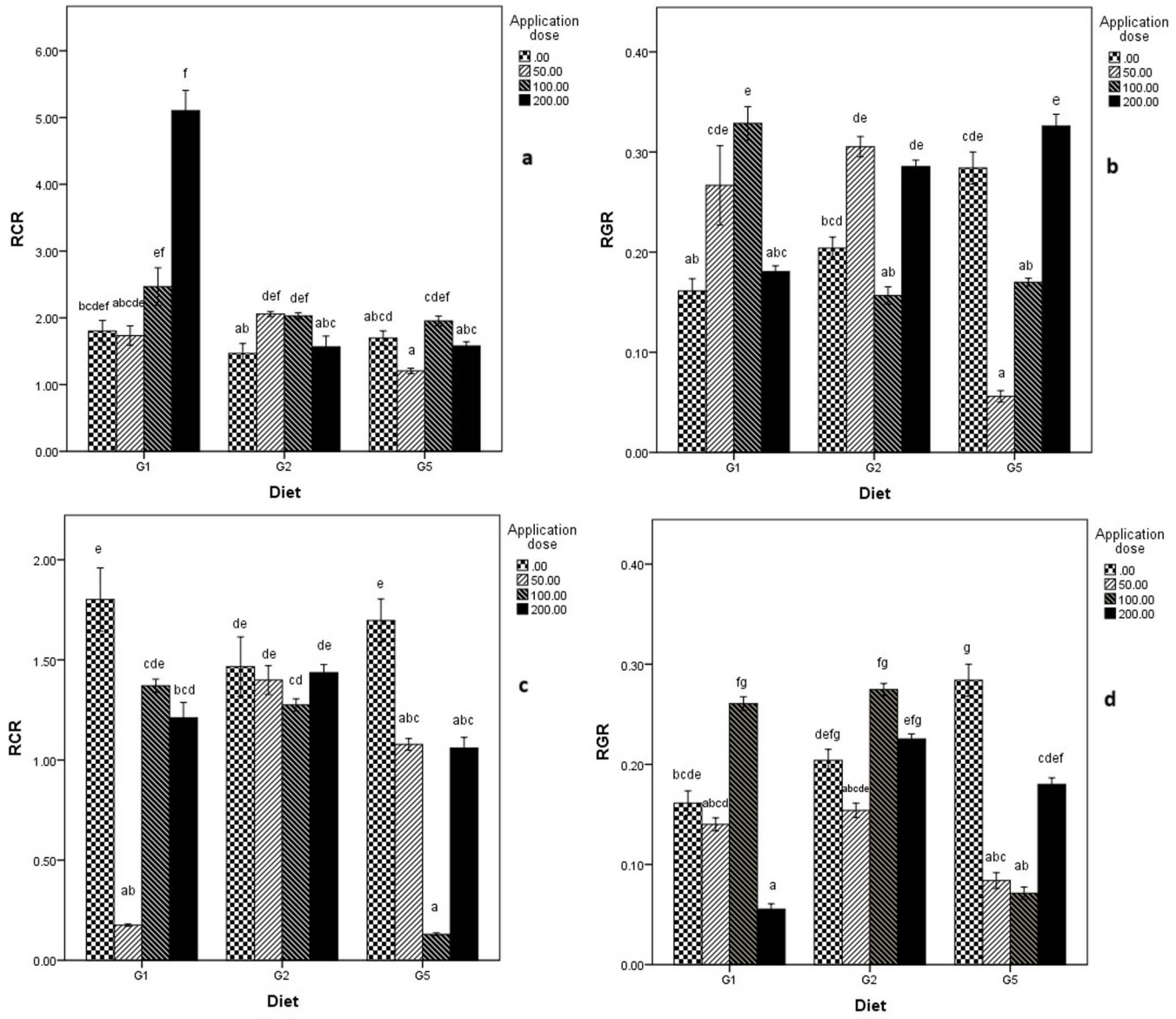


Figure 2. RCR and RGR of *C. perpectalis* larvae treated with different trichoderma strains (a, b) YP1A strain; (c, d) ID11D strain.
Note: Values in both strains differ between groups (RCR: YP1A; $X^2 = 129.38$, $df = 11$, $p < 0.01$; ID11D; $X^2 = 144.08$, $df = 11$, $p < 0.01$); RGR: YP1A; $X^2 = 149.30$, $df = 11$, $p < 0.01$; ID11D; $X^2 = 169.60$, $df = 11$, $p < 0.01$).

However, the RCR value decreases as the applied dose increases for the ID11D strain (table 4).

The lowest RGR values were found in larvae fed the G5 diet treated with 50 µL of fungal suspension for strain YP1A and the G1 diet treated with 200 µL of fungal suspension for strain ID11D. The highest values were found in larvae fed on the YP1A100 and G5 diets treated with 200 µL of fungal suspension for strain YP1A. The highest value was found for strain ID11D fed on the G5 diet (fig. 2). In the treated larvae with strain YP1A, the amount of nitrogen and application dosage affected the RGR value (two-way ANOVA, $df = 6$, $F = 255.44$, $p < 0.01$). In the ID11D strain, it was found that nitrogen level and application dose together affected the RGR value in the treated larvae (two-way ANOVA $df = 6$, $F = 402.27$, $p < 0.01$). In the YP1A strain, the RGR value increased with increasing application dose; in the ID11D strain, the RGR value was found to decrease with increasing application dose. No relationship was found between the amount of nitrogen and the RGR value in either strain (table 4).

The lowest pupal mass was found in the untreated G2 diets for both strains. The highest pupal weights were in the G1 diet treated with 50 µL of fungal suspension for strain YP1A and in the G2 diet treated with 50 µL of fungal suspension for strain ID11D (fig. 3). It was found that nitrogen level and application dose together affected pupal mass for strain YP1A (two-way ANOVA, $df = 6$, $F = 130.59$, $p < 0.01$). While pupal mass decreased with increasing nitrogen level, no relationship was found between the application dose and the pupal mass (table 4). In the ID11D strain, the amount of nitrogen and application dose affected the pupal mass (two-way ANOVA, $df = 6$, $F = 78.23$, $p < 0.01$). While there was no relationship between nitrogen level and pupal mass, pupal mass increased with the increasing application dose (table 4).

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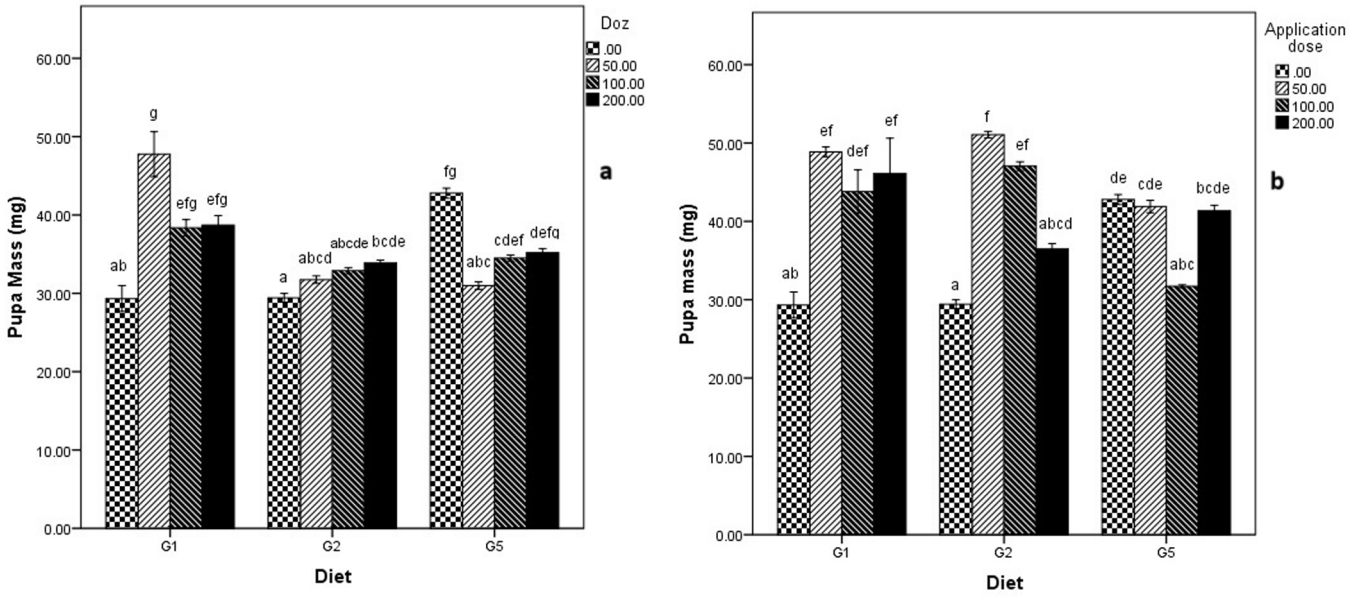


Figure 3. (a) Pupal mass of larvae treated with IYP1A strain (mean ± standard error). (b) Pupal mass of larvae treated with ID11D strain (mean ± standard error).
Note: Different letters indicate differences between groups. *$p < 0.01$.

Enzyme activities

The lowest PO activity in both strains was found in the G5 diet without treatment. In contrast, the highest activities were detected in larvae fed the G1 diet treatment at 200 µL of fungal suspension for YP1A strain and the G5 diet treatment at 200 µL of fungal suspension for strain ID11D (figs. 4 and 5). Nitrogen level and application dose affect PO activity in strains YP1A and ID11D (respectively; two-way ANOVA, $df = 6$, $F = 16292.41$, $p < 0.01$; $df = 6$, $F = 40704.92$, $p < 0.01$). In larvae treated with the strain YP1A, PO activity decreased with increasing nitrogen level, and activity increased with increasing application dose. In larvae treated with the strain ID11D, nitrogen has no effect on PO activity. However, the activity increased with increasing application dose (table 4).

The lowest SOD activities were found on the G2 diet treatment of 50 µL for strain YP1A and the G1 diet treatment of 50 µL of fungal suspension for strain ID11D. The highest SOD activities were found on the G5 diet treatment of 100 µL for strain YP1A and the G2 diet treatment at 200 µL of fungal suspension for strain ID11D (figs. 4 and 5). SOD activity was higher in the YP1A treatment than in the ID11D treatment (figs. 4 and 5). In both strains, nitrogen and dose together affect SOD activity (two-way ANOVA, YP1A, $df = 6$, $F = 349{,}250.45$, $p < 0.01$; ID11D, $df = 6$, $F = 58{,}593.81$, $p < 0.01$). SOD activity increases with increasing nitrogen levels in larvae treated with strain YP1A. However, no relationship between dose and activity was found. In strain ID11D, the activity increases with increasing nitrogen level and the application dose (table 4).

The lowest CAT activities were found on the G5 diet treatment with YP1A strain and the G1 diet treatment of 100 µL of fungal suspension for strain ID11D. The highest activities were found on the G2 diet treatment of 200 µL of fungal suspension for strain YP1A and the G2 diet treatment of 200 µL of fungal suspension for strain ID11D (figs. 4 and 5). When comparing strains YP1A and ID11D, the CAT activity of strain ID11D is higher (figs. 4 and 5). In both strains, YP1A and ID11D, The amount of nitrogen and the dose affect the CAT activity (respectively, two-way ANOVA, $df = 6$, $F = 112065.93$, $p < 0.01$; $df = 6$, $F = 200324.69$, $p < 0.01$). It was observed that CAT activity increased with increasing dose in the treatment of both strains. However, no relationship was found between the amount of nitrogen and CAT activity in either strain (table 4).

It was found that the lowest GPx activities were on the G2 treatment of 50 µL for strain YP1A and the G5 diet treatment of 50 µL of fungal suspension for strain ID11D. The highest activities were on the G5 diet treatment of 200 µL of fungal suspension for strain YP1A and the G2 diet treatment of 200 µL of fungal suspension for strain ID11D (figs. 4 and 5). It was found that GPx activity was higher in the ID11D treatment than in the YP1A treatment (figs. 4 and 5). Nitrogen level and dose affected GPx activity in the strain YP1A (two-way ANOVA, $df = 6$, $F = 207.51$, $p < 0.01$). Increasing nitrogen level and dose increased GPx activity (table 4). In strain ID11D, nitrogen level and dose affected GPx activity (two-way ANOVA, $df = 6$, $F = 4098.33$, $p < 0.01$). As nitrogen level increased, GPx activity decreased, and as dose increased, activity increased (table 4).

Mortality rate

Mortality rates of larvae in the different diet groups corrected by the Sun–Sheppard formula are given in table 5. Accordingly, the highest mortality rate was recorded in the G5 diet treatment with 200 µL of fungal suspension of ID11D, and the lowest mortality rate was recorded in the larvae fed on the G2 diet treatment with 50 µL of both fungal suspension. In strain YP1A, mortality decreased with increasing nitrogen levels and increased with increasing dose levels. However, in contrast to strain YP1A, the amount of nitrogen had no effect on mortality, but the mortality rate increased with increasing dose (table 4).

Discussion

The most important way to prevent the damage caused by insect pests in agricultural and forest ecosystems is to have information on effective methods of control. Just as *C. perspectalis* larvae feed

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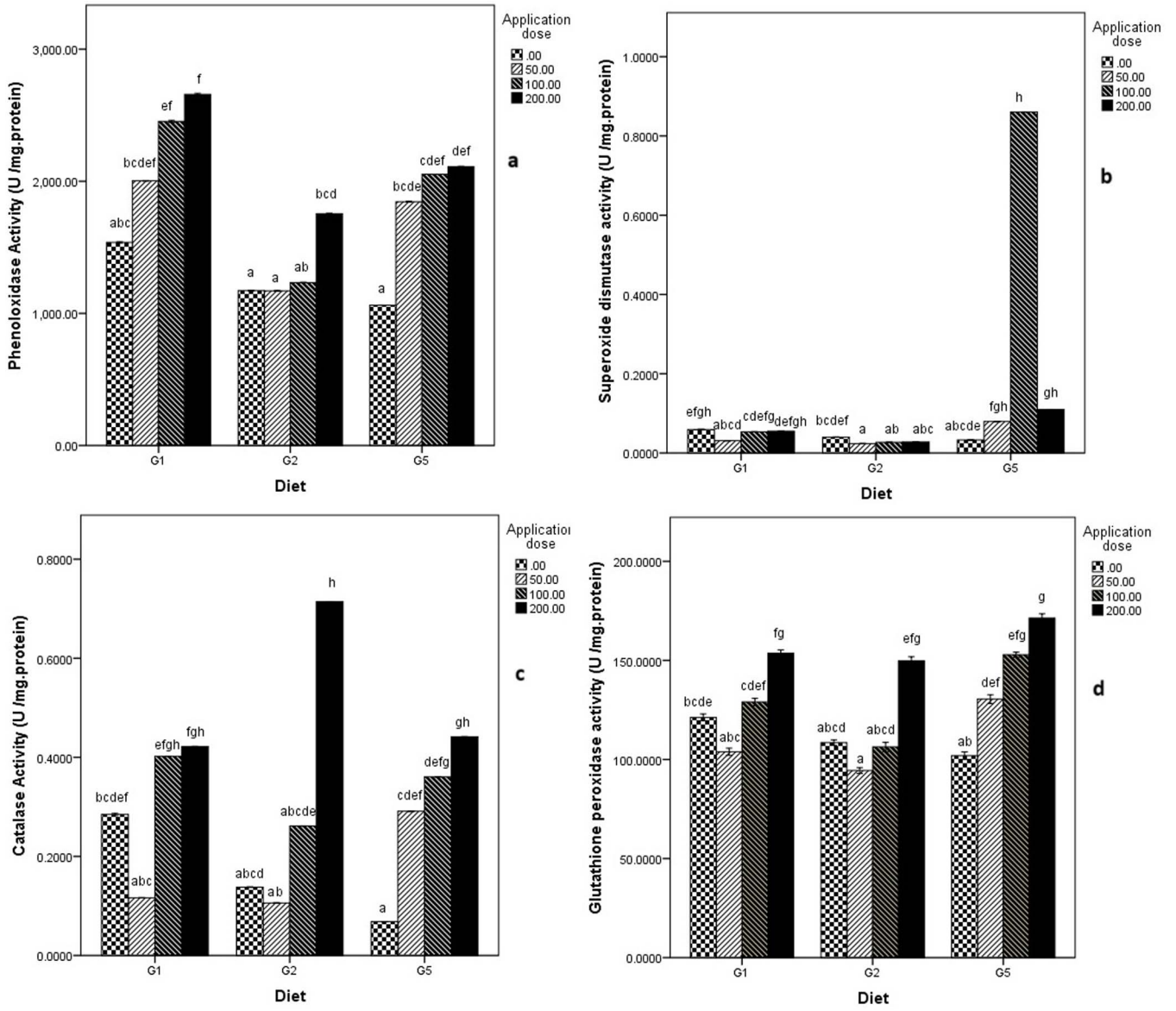


Figure 4. (a–d) Enzyme activities of larvae treated with YP1A strain (mean ± standard error).
Note: *Phenoloxidase: $X^2 = 177.32$, $df = 11$, $p < 0.01$. SOD: $X^2 = 177.30$, $df = 11$, $p < 0.01$; CAT: $X^2 = 177.79$, $df = 11$, $p < 0.01$.

on naturally growing boxwood, they cause significant economic damage to boxwood seedlings grown for landscaping purposes in parks and gardens (Ok *et al.*, 2023). This study shows that ID11D and YP1A strains differentially affect larval food use efficiency and enzyme activities. *Trichoderma* isolates can promote plant growth due to their properties, such as rapid growth rates and antimicrobial metabolites. *Trichoderma* isolates are also an important antagonistic fungal agent against pests (Verma *et al.*, 2007). *T. harzianum* causes larval death by acting on the peritrophic membrane in the intestine when taken orally with its chitinase enzyme (Berini *et al.*, 2016; Poveda, 2021). In addition, different isolates of entomopathogenic fungi may have different efficacy against the same species due to their characteristics, such as the secondary metabolites they possess and the activity of their conidia (Graf *et al.*, 2023). Therefore, the differential efficacy of different strains on *C. perspectalis* larvae is an expected result.

Host plant quality is one of the factors that influence nutritional indices and one of indicators of the plant quality is the amount of nitrogen in plant material. Consumption and food availability are the main factors that influence the reproduction, development, and adult activity of insect (Nandhini *et al.*, 2023). ECD and ECI, among the nutritional indices, indicate the insects's ability to digest and consume food (Nathan and Kalaivani, 2005). Altered ECD and ECI values indicate changes in the utilisation of digested and ingested nutrients for body mass (Abdel-Rahman and Al-Mozini, 2007). For both applied strains, the AD values of larvae fed on plants with different nitrogen levels did not show any differences. However, the ECD and ECI values of larvae treated with the ID11D strain were higher than those of larvae treated with the YP1A strain. However, RCR and RGR were found to be higher in the YP1A strain. The reason for the high ECD and ECI values in the ID11D strain, while RCR and RGR values were low in the same

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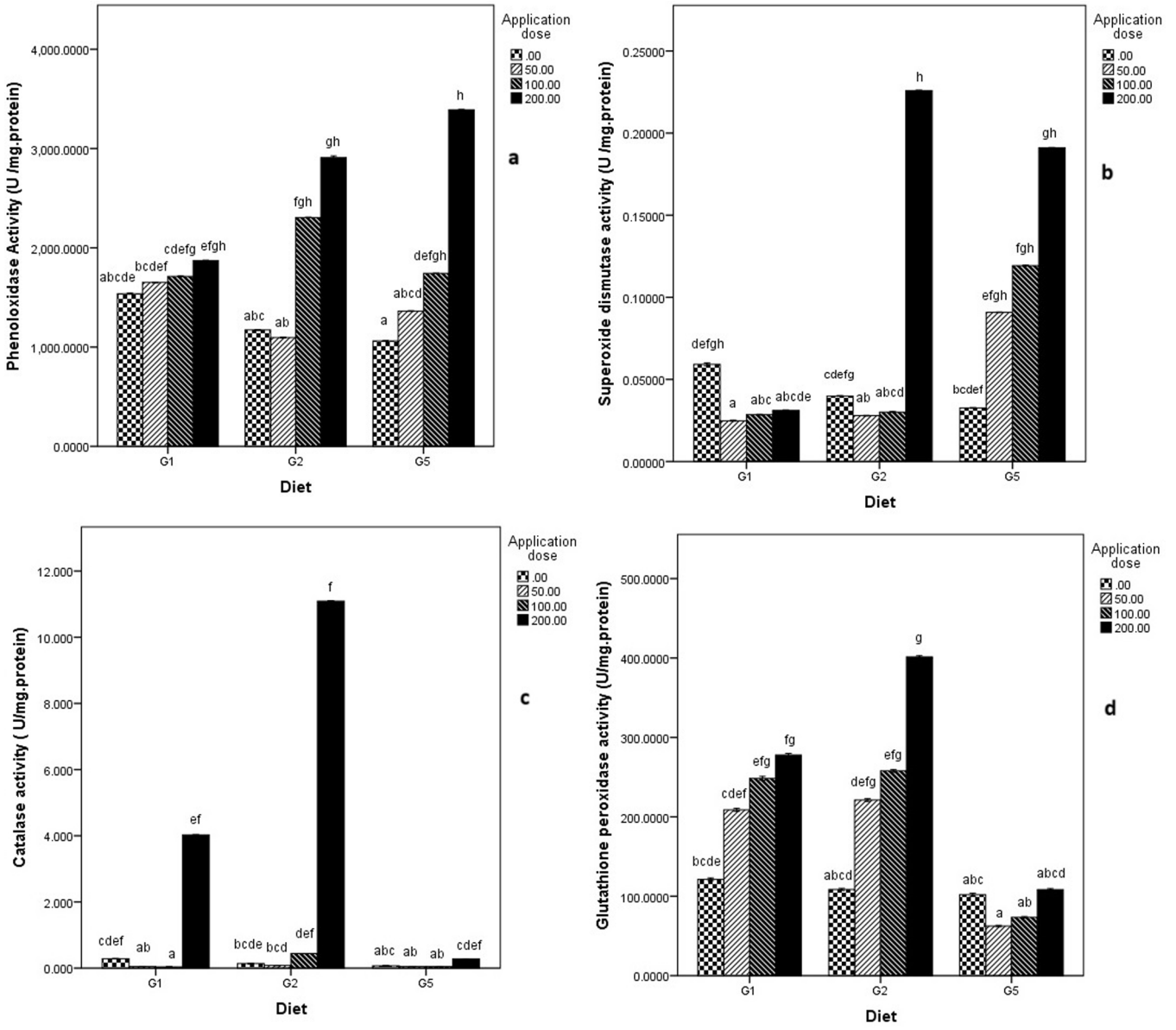


Figure 5. (a–d) Enzyme activities of larvae treated with ID11D strain (mean ± standard error).
Note: *Phenoloxidase: $X^2 = 177.38$, $df = 11$, $p < 0.01$. SOD: ID11D; $X^2 = 177.14$, $df = 11$, $p < 0.01$. CAT: $X^2 = 175.35$, $df = 11$, $p < 0.01$.

strain, indicates that the digested food could not be utilised for growth. Another noteworthy result is that although no relationship was found between ECD and ECI indices and dose in the YP1A strain, such a relationship was observed in the ID11D strain. The decrease in RCR and RGR values with the ID11D dose indicates that the ID11D strain may be effective in the biocontrol of *C. perspectalis* larvae. Feeding indices, for instance RGR, RCR, are vital indicators for recognising resistance in selected crops and executing pest management tactics (Ajmal *et al.*, 2024). The decrease in RCR and RGR values may be because *Trichoderma* damages the insect cuticle and peritrophic membrane, resulting in decreased digestibility and availability of nutrients (Sarkhandia *et al.*, 2023). In addition, damage to this region and the cuticle makes the insect more permeable to pathogens (Berini *et al.*, 2016). The decreased RCR and RGR values mean that nutrients cannot be used sufficiently for vital events.

Fertilisation affects the morphology and physiology of the plant. The development time of the plant, the size of the plant parts, and the phytochemical properties of the plants can be altered by fertilisation. As a result, these changes may affect the use of the plant by pests (Hwang and Lauenroth, 2008). In this study, the nitrogen content of the leaves was changed by fertilisation. This led to changes in larval nutritional indices and enzyme activities when different strains were used. It was found that ECD and RCR values decreased with increasing nitrogen in YP1A, while ECI and ECD values increased with increasing nitrogen in ID11D. It is known that the low nitrogen content of host plants reduces the performance of larvae. For example, it has been found that adult individuals of the species *Pieris rapae* prefer to lay eggs on plants whose nitrogen content has been increased by fertiliser application (Chen *et al.*, 2004). Similarly, *Lymantria dispar* larvae were found to have higher ECD and ECI values in diets with higher nitrogen

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Table 5. Sun–Sheppard formula-corrected mortality rates of larvae fed in the experimental groups described in table 1

Diet-strain	Mortality (%)	Diet-strain	Mortality (%)
G1	7.0 ± 0.13[b]	G1	7 ± 0.13[b]
YP1A50	11.76 ± 0.023[c]	ID11D50	23.27 ± 0.16[c]
YP1A100	15.96 ± 0.03[d]	ID11D100	15.96 ± 0.09[a]
YP1A200	32.13 ± 0.07[e]	ID11D200	20.45 ± 0.12[d]
G2	19.78 ± 0.06[f]	G2	19.78 ± 0.06[e]
G2YP1A50	1.96 ± 0.02[a]	G2 ID11D50	1.96 ± 0.03[f]
G2YP1A100	26.47 ± 0.1[g]	G2 ID11D100	9.51 ± 0.08[g]
G2YP1A200	21.56 ± 0.09[h]	G2 ID11D200	25.12 ± 0.08[h]
G5	7.12 ± 0.03[j]	G5	7.12 ± 0.03[k]
G5YP1A50	5.12 ± 0.05[k]	G5 ID11D50	15.96 ± 0.09[a]
G5YP1A100	1.96 ± 0.02[a]	G5 ID11D100	3.74 ± 0.04[m]
G5YP1A200	7.74 ± 0.05[m]	G5 ID11D200	32.13 ± 0.08[n]

Note: Different letters indicate differences between different groups (each strain was evaluated on its own).

content (Stockhoff, 1992). Therefore, in larvae fed on diets treated with YP1A, the decrease in ECD and RCR with increasing dietary nitrogen may be due to the inability of the larvae to utilise the food efficiently. In sugarcane plants, nitrogen fertilisation together with *Trichoderma harzianum* reduced the nutrient use efficiency of *Sclerotium rolfsii* individuals (Khattabi *et al.*, 2004). Therefore, nitrogen fertilisation and ID11D application in boxwood seedling cultivation may reduce the nutrient utilisation efficiency of larvae. These results are also supported by the mortality rates. In the YP1A application, larvae fed with nitrogen-rich food have a low mortality rate.

In *Spodoptera littoralis* when larvae were fed on *Solanum lycopersicum* leaves treated with *Trichoderma*, an imbalance in the metabolic activities of the gut microbiota was observed and pupal mass was reduced by the fungal toxins (Monte, 2023). However, this study found that the pupal mass of *C. perspectalis* increased with increasing dose in the ID11D strain, whereas there was no relationship between the dose and pupal mass in the YP1A strain, which differs from the literature. Although pupal mass is considered an indicator of fecundity (Honěk, 1993), it is not the only determining factor.

PO activity and inhibition in invertebrates is a complex system. Phenol oxidase, which plays an essential role in melanogenesis, converts phenols into quinones. These are then polymerised to form melanin. Melanin plays a role in pigmentation of invertebrates. Melanin accumulation has been found to increase in the presence of pathogens, mechanical damage, and foreign substances (González-Santoyo and Córdoba-Aguilar, 2012). In *S. litura* larvae infected with *B. bassiana*, PO activity was found to increase 24 h after infection (Bali *et al.*, 2013). PO activities were found to increase 8–12 h after infection in *Heterorhabditis bacteriophora* larvae infected with *Agriotes lineatus* (Rahatkhah *et al.*, 2015). In infected *C. perspectalis* larvae in this study, an increase in PO activity was observed for both strains and with increasing dose, which is in agreement with the literature. However, it was found that the PO activities of larvae infected with the ID11D strain were higher than those infected with YP1A. In this case, the ID11D strain triggers the immune response of the larvae more than the YP1A strain.

Specific components of the insect immune system produce ROS to limit microbial growth. Insects produce several antioxidant and detoxification enzymes to scavenge free radicals. The main antioxidant enzymes are SOD, GPx, and CAT. SOD scavenges and detoxifies superoxide anions by converting them to hydrogen peroxide and oxygen. Hydrogen peroxide is converted to water and oxygen by CAT and GPx (Lalitha *et al.*, 2018). In this study, a significant increase in SOD, CAT, and GPx activities was observed in *C. perspectalis* larvae, especially with the 200 µL of fungal suspension application. However, this increase is higher in the ID11D strain than in the YP1A strain, which is consistent with the literature in this regard (Li *et al.*, 2016; Shahriari *et al.*, 2023). The increase in SOD and CAT enzyme activities after infection has been associated with excessive responses and programmed cell death in tissues. The oxidative burst that causes the increase has been linked to pathogen-induced host death (Schenk *et al.*, 2000). Paes *et al.* (2001) stated that there are changes in antioxidant enzyme activities with oxidative stress in different systems. The higher activity in individuals infected with the ID11D strain compared to the YP1A strain after infection is associated with more oxidative stress in *C. perspectalis* larvae after infection. In addition, this difference may indicate the infectivity of the host by the strains (Simoes and Rosa, 1996).

As a result, different strains of the same species have different activity against *C. perspectalis* larvae. It was found that the ID11D strain was more effective in areas where nitrogen fertiliser was applied, and the YP1A strain was more effective in areas where nitrogen fertiliser was not applied. In addition, both strains induce the immune and antioxidant systems of *C. perspectalis* larvae. Although laboratory studies have shown that the ID11D strain is more effective than YP1A strain against larvae feeding on fertilised boxwood, further field trials are needed to control *C. perspectalis* larvae. In field trials, both strains can be view as potential candidates for biological control of *C. perspectalis* larvae on plants with different fertilisation.

Acknowledgements. This study is based on the master thesis on Recep Tayyip Erdoğan University. Furthermore, it was presented as an oral presentation at the 1st International Boxwood Workshop, Recep Tayyip Erdoğan University, Rize, Turkey, 25–27 October 2021, and it was published as a summary abstract in the proceedings book.

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