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Pathogenicity of Two Fungal Strains, *Metarhizium brunneum* (ORP-18) and *Beauveria bassiana* (GOPT-331), against Larvae of the European Tent Caterpillar *Malacosoma neustria* (L., 1758) (Lepidoptera: Lasiocampidae)

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Abstract: *Malacosoma neustria* is a pest for many plant species. Instead of chemical control, eco-friendly biological control methods should be used to combat this species. The purpose of this study was to evaluate the efficacy of isolates of two entomopathogenic fungi, *Metarhizium brunneum* (ORP-18) and *Beauveria bassiana* (GOPT-331), used as both oral and spray application on the 4th instar larvae of *M. neustria* under laboratory conditions. In addition, it was determined how the infection affected the amounts of malondialdehyde, NADPH oxidase and glutathione of the larvae. Both fungal isolates were placed in separate containers at 2 ml for each concentration (1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml) in the oral group and contaminated all over the *Elaeagnus rhamnoides* leaves and then the contaminated leaves were offered to ten larvae in each container. For the spray group, 2 ml of spray was applied to every ten larvae placed in the containers for each concentration. As a result, it was found that both isolates killed the larvae. The ORP-18 isolate caused higher mortality rates than the GOPT-331 isolate. The spray application was more lethal than the oral application. With increasing conidial concentrations of both isolates, the amounts of malondialdehyde and NADPH oxidases increased, while the glutathione amounts decreased, which indicated the adverse effects of reactive oxygen species (ROS) on larval survival. On the basis of the results, it is recommended to use the ORP-18 isolate for the control of larvae of *M. neustria*.

Key words: *Beauveria bassiana,* glutathione, *Malacosoma neustria,* malondialdehyde, *Metarhizium brunneum*, NADPH oxidase

Introduction

The European tent caterpillar *Malacosoma neustria* (Linnaeus, 1758) (Lepidoptera: Lasiocampidae) frequently causes severe damage to economically important fruit trees such as plums, apples, hazelnuts and pears as well as to wild shrubs and ornamental trees (Ozbek & Çalmasur 2005, Ozbek & Çoruh 2010). Newly hatched caterpillars migrate to new branches, feed first on buds, then the upper

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epidermis and finally the parenchyma of leaf tissue. In some years, the host plants become completely leafless due to the high number of the pest (Gençer et al. 2019), so combating this organism is critical. Biological control methods should be used instead of synthetic insecticides due to their harmful effects on non-target organisms, the environment and human health as well as due to the development of insect resistance (Abriham et al. 2018). The use of entomopathogenic microorganisms as biological control agents is essential in combating harmful species in terms of both targeting a specific pest and also being eco-friendly (Abriham et al. 2018). Entomopathogenic fungi (EPF) are organisms used in this context. EPF infect insects of various orders, especially Hemiptera, Diptera, Coleoptera, Lepidoptera, Orthoptera and Hymenoptera (Ramanujam et al. 2014). Members of the genera *Beauveria* and *Metarhizium* are ubiquitous in almost all ecosystems and are among the most studied insect pathogenic fungi (Boucias et al. 2018). Many studies have shown the effects of these fungi against various insects (e.g. Inanli et al. 2012, Lacey et al. 2015, van Lenteren et al. 2018).

Numerous pathogens to which insects are exposed during their lifetime cause the induction of oxidative stress. Oxidative stress can be defined as an imbalance between the production of reactive oxygen species (ROS) and the organism's ability to neutralise the damage they cause (Kramer et al. 2021). ROS is naturally produced in all cells and organisms but excessive production can cause oxidative stress by damaging important biomolecules such as proteins, lipids or nucleic acids (Selman et al. 2012). One of the processes studied in connection with the effects of ROS is lipid peroxidation. Lipid peroxidation, the oxidation process of unsaturated free fatty acids (Gaschler & Stockwell 2017), induces cellular damage and is used as an indicator of oxidative stress. The malondialdehyde (MDA) amount is generally an indicator of lipid peroxidation and reflects oxidative stress (Kazek et al. 2020). Infection by fungal pathogens changes the amounts of MDA in insects (Chaurasia et al. 2016, Kazek et al. 2020). NADPH oxidases (NOx) are another leading source of ROS (Moghadam et al. 2021). NOx is expressed in various cell and tissue lines and is present in most eukaryotes (Bedard et al. 2007, Aguirre & Lambeth 2010). Fungal infection causes the formation of ROS, especially NOx, as a result of metabolic processes (Aguirre et al. 2005). To overcome these adverse conditions, the antioxidant capacity of insects uses non-enzymatic ROS scavengers such as glutathione (GSH) (Cen et al. 2020). Besides protecting insects from oxidative stress, GSH plays an essential role in insect immunity through the detoxification and metabolism of toxins in the insect body (Kumar et al. 2003).

In this study, the efficacy of EPF *Metarhizium brunneum* (isolate ORP-18) and *Beauveria bassiana* (isolate GOPT-331) isolates against the 4th instar larvae of *M. neustria* was evaluated under laboratory conditions. The effects of these isolates on larval survival were investigated. In addition, it was aimed to evaluate oxidative stress caused by the fungal infection determined by the amounts of MDA, NOx and GSH in the larvae.

Materials and Methods

Insect sampling

Malacosoma neustria eggs were collected from *Elaeagnus rhamnoides* L. plants at the Kızılırmak Delta of Samsun Province, Turkey (41°30'N; 36°05'E), in May 2020 and brought to the laboratory. They were disinfected for about 7 min by 10 % sodium hypochlorite and then washed for about 7 min with distilled water. The disinfected eggs were at 70 % RH, and 24 °C (16 hrs. light/8 hrs. dark).

Fungal cultures

The EPF *M. brunneum* (isolate ORP-18) and *B. bassiana* (isolate GOPT-331) originating from soil samples from Tokat and Ordu Province were collected and identified by Dr Yusuf Yanar. These isolates were tested in the study. For identification of the isolates, DNA extractions of fungi were performed. Amplification of genomic DNA by PCR was carried out using ITS4/ITS5 primers. The isolates were diagnosed by sequence analysis and recorded at the GenBank database (Accession number of ORP-18: MW410200 and GOPT-331: MK411548). The isolates had previously been tested for pathogenicity and were considered to be virulent. They were grown on potato dextrose agar (PDA) medium in an incubator at 24±2°C and a 16-h photoperiod for 15–30 days.

Preparation of conidial suspensions

Fungal conidia were harvested from sporulating cultures by scraping with a scalpel. The conidial suspension was prepared by adding 10 ml of sterile distilled water containing 0.02 % Tween 80. The conidial suspension was vortexed for 1–2 min and filtered through four layers of sterile cheesecloths to remove mycelial fragments. The spore suspensions were adjusted to the concentrations of 1×10^5 – 1×10^8 conidia/ml, using a hemocytometer. The viability of conidia was determined by applying 0.1 ml of the suspension to PDA plates. A sterile microscope coverslip was placed on each plate and incubated at 27 °C. The percentage of germination was examined by counting 100 spores per dish after 24 hrs.

Pathogenicity tests

Hatched larvae were fed on the leaves of *E. rhamnoides* (sterilised with 50 % ethyl alcohol and rinsed with distilled water). The 4th instar larvae were collected and put in plastic containers for the experiments. Different concentrations (1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml) of *M. brunneum* (ORP-18) and *B. bassiana* (GOPT-331) isolates were added in separate containers, at 2 ml from each concentration in the oral group, and contaminated by spraying all over the disinfected *E. rhamnoides* leaves. The contaminated leaves were placed in the containers, and ten larvae of the 4th instar were placed in each container. For the spray group, 2 ml of spore suspension was applied, using an atomiser for every group of ten larvae placed in the containers at each concentration (1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml). In the control groups, the larvae were fed on the disinfected leaves. The experiment was carried out in five replicates for each group. All containers were incubated at 24 °C, 70 % RH and a 16:8 h light : dark period. The larvae were observed for 25 days during the survival experiment.

Haemolymph sampling

For each group, 100 larvae were used to determine the MDA, NOx, and GSH amounts. After disinfecting larvae with 95 % ethanol, haemolymph samples were taken using a microcapillary tube by puncturing the third legs of the larvae with a fine-tipped dissecting needle and transferred to Eppendorf tubes containing N-phenylthiourea (Sigma-Aldrich) (Kara et al. 2020).

Determination of MDA

MDA amounts were measured by the method of Buege & Aust (1978). The haemolymph was diluted at 1:10 with phosphate buffer. As many as 0.5 ml of 5 % trichloroacetic acid (TCA) was added onto 1 ml of haemolymph for deproteinisation. The samples were then mixed and centrifuged for 10 min at 3000 RCF. To 1 ml sample or standard (1,1,3,3-tetraethoxy propane), 10 µl butyl hydroxy toluene (BHT, 1 %, w/v), 0.5 ml thiobarbituric acid (TBA, 0.67 %, w/v) were added. Samples were mixed and incubated in a boiling water bath for 10 min. The absorbance of samples or standard was measured spectrophotometrically at 535 nm. MDA amount was calculated using the formula "(ABS of sample*standard concentration)/ABS of the standard" and expressed as nanomol per millilitre of haemolymph.

Determination of NOx

NOx amounts were measured by the method of Miranda et al. (2001). The haemolymph was diluted at 1:10 with deionised water. The haemolymph (1 ml) was taken and added to 0.5 ml of 0.3 M NaOH. As many as 0.5 ml of 10 % (w/v) $ZnSO_4$ was added onto the mixture for deproteinisation after incubation for 5 min at room temperature. The samples were then centrifuged at 14000 RCF for 5 min. Nitrate concentrations of supernatants were measured spectrophotometrically, based on the reduction of nitrate to nitrite by vanadium chloride ($VaCI_3$). Then, nitrite concentrations were determined by the Griess reaction. NOx (nitrite + nitrate) concentration was calculated from the calibration curve of sodium nitrite standards and expressed as nanomol per millilitre of haemolymph.

Determination of total GSH

Total GSH was measured by the modified method of Aykaç et al. (1985). The haemolymph was diluted at 1:10 with phosphate buffer. Trichloroacetic acid (TCA, 0.2 ml, 5 %) was added onto 0.6 ml of haemolymph for deproteinisation. The samples were then mixed and centrifuged for 10 min at 3500 RPM. After centrifugation of mixtures, 3 ml of 0.3 M phosphate buffer (pH 7.4) was put onto 0.6 ml of supernatant. After then, a 0.4 ml solution of dithiobisnitrobenzoate (DTNB, 0.3 mg/ml) was added to the samples. The absorbance of samples was measured spectrophotometrically at 412 nm. GSH concentration was calculated using the coefficient of 13,600 mol^{-1} cm^{-1} and expressed as micromoles per millilitre of haemolymph.

Statistical analyses

Cox-Regression analysis was used to calculate the risks of death of *M. neustria* larvae exposed to different fungal concentrations of both isolates for spray and oral applications. Probit analysis was used to calculate the lethal doses (LC_{50}). ANOVA Dunnet test was used to compare the amounts of MDA, NOx, and GSH compared to the control group. SPSS 21.0 software was used for statistical analyses.

Results

According to the Cox-Regression analysis results, there was a statistical difference ($P < 0.01$) between the control group and each of the groups infected with GOPT-331 and ORP-18 isolates (Table 1). The GOPT-331 isolate was found to increase the risk of mortality 9.2-fold, while the ORP-18 isolate increased it by 17.6-fold (Table 1). Survival curves of the control and fungal groups (Fig. 1) demonstrated that the ORP-18 isolate caused more adverse effects on larval survival than the GOPT-331 isolate. When the spray and oral applications of both isolates (except the oral application of GOPT-331) were compared to the control group, it was found that there was a statistical difference ($P < 0.05$). Both isolates were found to cause a stronger lethal effect when applied as a spray. When the GOPT-331 isolate was applied as a spray, the risk of mortality increased 14.9-fold, while the oral application increased the risk by 4.3-fold. Such risk increased 12.8-fold in the ORP-18 isolate after the oral application and 21.6-fold after the spray application (Table 2).

Because the spray application resulted in a higher mortality rate than the oral application, the concentration data of both isolates according to the spray application were tested using Cox-Regression analysis. Compared to the control group, the difference in the risk of mortality at the 1×10^5, 1×10^6, and 1×10^7 conidia/ml concentrations of the GOPT-331 isolate was non-significant ($P > 0.05$), but there was significant difference at the 1×10^8 conidia/ml concentration. The difference in the risk of mortality at 1×10^5 and 1×10^6 conidia/ml concentrations of the ORP-18 isolate was non-significant ($P > 0.05$), but there was significant difference ($P < 0.05$) at 1×10^7 and 1×10^8 conidia/ml concentrations. While the risk of mortality increased approximately 30-fold at the highest conidia concentration of the GOPT-331 isolate (1×10^8 conidia/ml), it increased 47.2-fold with the application of the ORP-18 isolate at a concentration of 1×10^8 conidia/ml (Table 3). The risk of mortality increased with increasing conidia concentration of both isolates.

The LC_{50} values of both fungal isolates for the spray and oral applications are shown in Table 4. Spraying conidia of the ORP-18 isolate yielded the lowest LC_{50} value, while the oral application of the GOPT-331 isolate produced the highest LC_{50} value.

The MDA amount in the control group was statistically different from the other groups (ANOVA, Dunnet test, F = 680.8, df = 169, $P < 0.001$). The MDA amounts were higher than those of control at all concentrations of both spray and orally administered fungal isolates. It was determined that low concentrations of the ORP-18 isolate (1×10^5 and 1×10^6 conidia/ml) were statistically similar to high concentrations of GOPT-331 isolate (1×10^7 and 1×10^8 conidia/ml) (Fig. 2).

Table 1. Cox proportional hazard regression analysis results of *Malacosoma neustria* larvae after treatments with *Beauveria bassiana* (GOPT-331) and *Metarhizium brunneum* (ORP-18) isolates. All concentrations ($1\times10^5 – 1\times10^8$ conidia/ml) and spray and oral applications of fungal isolates shown together.

Groups	**B**	**SE**	**Wald**	**df**	**P**	**Exp (B)**	**95.0 % CI for Exp (B)**	
							Lower	**Upper**
Control			25.609	2	.000			
GOPT-331	2.222	1.007	4.868	1	.027	9.225	1.282	66.397
ORP-18	2.867	1.004	8.155	1	.004	17.592	2.458	125.892

B: Coefficient of regression, SE: Standard error, Wald: Significance of the regression coefficients, df: Degree of freedom, P: Significant, Exp (B): Hazard proportion, CI: Confidence interval.

Table 2. Cox-Regression analysis results of *Malacosoma neustria* larvae according to the spray and oral applications of *Beauveria bassiana* (GOPT-331) and *Metarhizium brunneum* (ORP-18) isolates. All concentrations ($1\times10^5 – 1\times10^8$ conidia/ml) of spray and oral applications of fungal isolates were shown together.

Groups	**B**	**SE**	**Wald**	**df**	**P**	**Exp (B)**	**95.0 % CI for Exp (B)**	
							Lower	**Upper**
Control			25.726	2	.000			
Spray (GOPT-331)	2.702	1.009	7.165	1	.007	14.903	2.012	105.11
Oral (GOPT -331)	1.456	1.029	2.003	1	.157	4.291	0.568	32.09
Spray (ORP-18)	3.073	1.007	9.317	1	.002	21.599	3.11	160.8
Oral (ORP-18)	2.552	1.011	6.375	1	.012	12.829	1.809	95.1

B: Coefficient of regression, SE: Standard error, Wald: Significance of the regression coefficients, df: Degree of freedom, P: Significant, Exp (B): Hazard proportion, CI: Confidence interval.

Table 3. Cox-Regression analysis results of *Malacosoma neustria* larvae according to the spray applications after treatments with different concentrations ($1\times10^5 - 1\times10^8$ conidia/ml) of *Beauveria bassiana* (GOPT-331) and *Metarhizium brunneum* (ORP-18) isolates.

Groups	B	SE	Wald	df	P	Exp (B)	95.0 % CI for Exp (B)	
							Lower	Upper
Control			69.297	4	.000			
1×10^5 conidia/ml (GOPT-331)	.414	1.155	.128	1	.720	1.512	.16	14.5
1×10^6 conidia/ml (GOPT-331)	.916	1.095	.699	1	.403	2.500	.29	21.3
1×10^7 conidia/ml (GOPT-331)	1.977	1.035	3.647	1	.056	7.219	.94	54.5
1×10^8 conidia/ml (GOPT-331)	3.398	1.010	11.309	1	.001	29.985	3.95	207.5
1×10^5 conidia/ml (ORP-18)	.400	1.155	.120	1	.729	1.491	.16	14.3
1×10^6 conidia/ml (ORP-18)	1.105	1.080	1.047	1	.306	3.020	.37	25.1
1×10^7 conidia/ml (ORP-18)	3.332	1.011	10.859	1	.001	28.003	4.04	212
1×10^8 conidia/ml (ORP-18)	3.855	1.008	14.633	1	.000	47.214	7.1	367

B: Coefficient of regression, SE: Standard error, Wald: Significance of the regression coefficients, df: Degree of freedom, P: Significant, Exp (B): Hazard proportion, CI: Confidence interval.

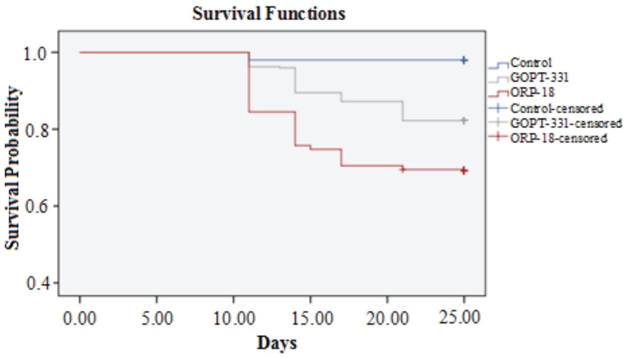

Fig. 1. Survival probability of *Malacosoma neustria* larvae after treatments with *Beauveria bassiana* (GOPT-331) and *Metarhizium brunneum* (ORP-18) isolates. All concentrations (1×10^5-1×10^8 conidia/ml) and spray and oral applications of fungal isolates were shown together.

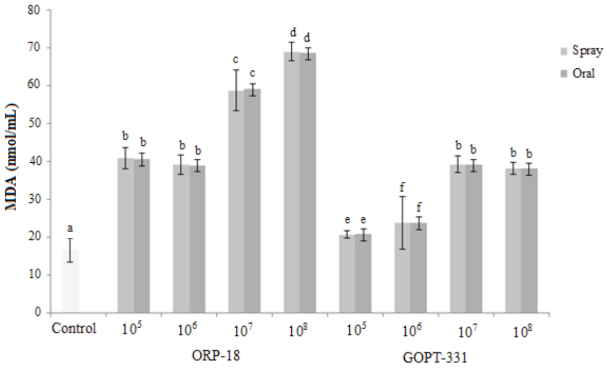

Fig. 2. Comparison of MDA amounts in haemolymph of *Malacosoma neustria* larvae after treatments with *Beauveria bassiana* (GOPT-331) and *Metarhizium brunneum* (ORP-18) isolates. Values are the means ±SE. Different letters on the error bars indicate significant differences. P = 0.05.

NOx amount of the control group (ANOVA, Dunnet test, F = 574.4, df = 169, $P < 0.001$) was statistically similar to the 1×10^5 and 1×10^6 conidia/ml groups of the GOPT-331 isolate applied both spray and orally. The highest NOx amounts were detected in larvae exposed to the highest concentration of the ORP-18 isolate, both spray and orally (Fig. 3).

The GSH amount of the control group was statistically different from the other groups (except for 1×10^5 conidia/ml concentration of the GOPT-331 isolate) (ANOVA, Dunnet test, F = 231.5, df = 169, $P < 0.001$). The GSH amount in the larvae exposed to both spray and oral applications were lower than those of control at all concentrations. It was found that low conidia concentrations of the ORP-18 isolate (1×10^5 and 1×10^6 conidia/ml) were statistically similar to high conidia concentrations of GOPT-331 isolate (1×10^7 and 1×10^8 conidia/ml) (Fig. 4).

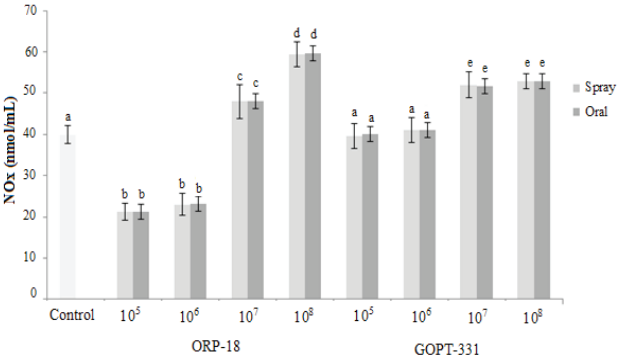

Fig. 3. Comparison of NOx amounts in haemolymph of *Malacosoma neustria* larvae after treatments with *Beauveria bassiana* (GOPT-331) and *Metarhizium brunneum* (ORP-18) isolates. Values are the means ±SE. Different letters on the error bars indicate significant differences. P = 0.05.

Table 4. Probit regression estimates for the multiple-concentration bioassays performed with *Beauveria bassiana* (GOPT-331) and *Metarhizium brunneum* (ORP-18) isolates for the spray and oral applications.

Isolate (treatment)	**LC$_{50}$ (conidia/ml) (*FL*, 95 %)**	**Intercept±SE**	**Slope±SE**	X^2	**df**	**95.0 % CI for Exp (B)**	
						Lower	**Upper**
GOPT-331 (oral)	2.0×10^{13}	-2.4±0.5	0.28±0.07	1.444	2	.04	.32
GOPT-331 (spray)	2.4×10^{8}	-4.3±0.7	0.5±0.1	9.061	2	.32	.71
ORP-18 (oral)	5.3×10^{7}	-3.9±0.6	0.6±0.08	2.925	2	.34	.66
ORP-18 (spray)	4.9×10^{6}	-5.5±0.7	0.8±0.1	17.764	3	.62	1.02

X^2: Chi-Square test, df: Degree of freedom, Exp (B): Hazard proportion, CI: Confidence interval

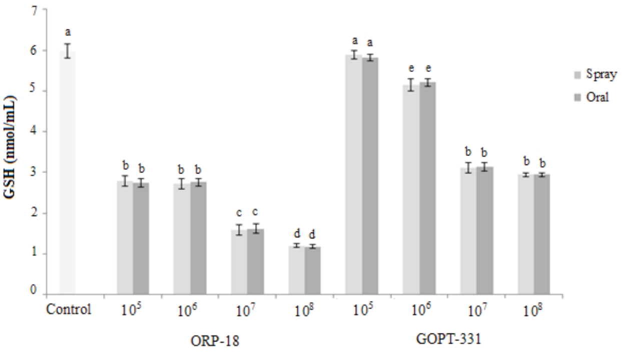

Fig. 4. Comparison of GSH amounts in haemolymph of *Malacosoma neustria* larvae after treatments with *Beauveria bassiana* (GOPT-331) and *Metarhizium brunneum* (ORP-18) isolates. Values are the means ±SE. Different letters on the error bars indicate significant differences. P = 0.05.

Discussion

Biological control of insect pests with EPF is one of the applications that can be used as an alternative to chemical insecticides. In terms of pest control effectiveness, EPF *Beauveria* and *Metarhizium* are the most preferred. In the present study, the efficacy of different isolates (ORP-18 and GOPT-331) of the two fungi against *M. neustria* larvae, both with spray and oral applications, was demonstrated. Both biocontrol agents showed mortality to *M. neustria* larvae.

The efficacy of different isolates of fungi used in our study has been investigated against various pests in several studies. Tkaczuk & Miętkiewski (1998) determined that EPF caused a reduction in the pine-tree lappet moth *Dendrolimus pini* (Linnaeus, 1758) population. In a study by Matek & Pernek (2018), it was determined that *B. bassiana* caused more than 98 % mortality in *D. pini*. Ge et al. (2009) demonstrated the effect of *B. bassiana* on the masson-pine caterpillar *D. punctatus* (Walker, 1855). Freed et al. (2012) also reported that high EPF concentration against *P. xylostella* led to a significantly higher mortality rate compared to low ones. Mehinto et al. (2014) in their study in which they applied *M. brunneum* and *B. bassiana* isolates to *Maruca vitrata* (Fabricius, 1787), noted that larval mortality increased with increasing dose for both isolates. Our finding that increased conidial concentration increased the larval mortality is consistent with these results.

Metarhizium brunneum isolates applied to *Tuta absoluta* (Meyrick, 1917) caused 86–95 % mortality rates (Akutse et al. 2020). Mkiga et al. (2020) in their study in which they infected *Thaumatotibia leucotreta* (Meyrick, 1913) with *M. brunneum* and *B bassiana* isolates, showed that 12 *M. brunneum* isolates caused a mortality rate of 58–94 %, three *B. bassiana* isolates caused a mortality rate of 57–84 %. In this study, both fungal treatments caused the death of *M. neustria* larvae and the *M. brunneum* ORP-18 isolate was more lethal than the *B. bassiana* GOPT-331 isolate.

Ozturk et al. (2015) used a spray method to test the pathogenicity of three *B. bassiana* isolates against different stages of *Leptinotarsa decemlineata* (Say, 1824) at 1×10^8 conidia/ml concentration. According to their findings, the tested isolates caused mortality rates ranging from 57.1 to 100 % in young (2nd and 3rd instar) larvae on the 3rd and 7th days after treatment, 36.7 to 100 % in old (4th instar) larvae, and 23.3 to 86.2 % in 1-week-old adults. Shehzad et al. (2021), in their study in which they infected *P. xylostella* with *M. brunneum* and *B. bassiana* isolates as spray and leaf dipping applications, stated that spray application caused more deaths and this method was more effective. The result of this study is consistent with our findings. In our study, it was found that the spray application for both isolates caused more mortality than contamination of *E. rhamnoides* leaves with the isolates. The spray application led to higher mortality in *M. neustria* larvae than the oral application because the spores were more likely to get in contact with the insect's body in the spray application.

When the LC$_{50}$ values were compared, it was determined that spraying ORP-18 isolate yielded the lowest value, while the oral application of the GOPT-331 isolate yielded the highest value. Probably digestive enzymes secreted in insect stomachs may cause the death of infectious spores of EPF. This may reduce the efficacy of oral application of EPF. The spray applications revealed lower LC_{50} values for both isolates. This finding indicated that the spray application would be preferable for *M. neustria* larvae control.

The MDA amounts increased as a result of both spray and oral treatments of both fungal isolates compared to the control. Similar results were obtained in other studies. For example, Shamakhi et al. (2018) noted that infection of *Chilo suppressalis* (Walker, 1863) with *B. bassiana* increased the MDA amounts compared to the control. Kaur et al. (2021) showed that fungal application to *Spodoptera litura* (Fabricius, 1775) larvae increased the level of lipid peroxidation compared to the control group. In this study, the MDA amounts increased with increasing conidial concentrations of both isolates (except for 1×10^6 conidia/ml concentration of the ORP-18 isolate). In this case, it can be said that the increase in conidial concentration causes more lipid peroxidation. The highest MDA amounts were observed in larvae exposed to 1×10^8 conidia/ml concentrations of the ORP-18 isolate. Thus, oxidative stress was maximal in larvae exposed to the highest conidia concentration of this isolate.

NOx has been shown to cause ROS production in insects (Sajjadian & Kim 2020). In this study, the NOx amounts of larvae exposed to both spray and oral treatments of both fungal isolates increased with increasing conidial concentration. It was determined that the NOx amounts of larvae exposed to high conidia concentrations of both isolates (1×10^7 and 1×10^8 conidia/ml) were higher than the control. This indicates that higher conidial concentrations cause more ROS formation. The fungal infection triggers the formation of ROS with NOx activity (Aguirre et al. 2005). NOx expression can be induced by lactic acid produced by the anaerobic metabolism of pathogens (Iatsenko et al. 2018). The activation of this enzyme indicates that toxic ROS production should be eliminated.

GSH in the haemolymph may be a mechanism of infection tolerance (Stahlschmidt et al. 2015) and is significant in eliminating ROS. In this study, the GSH amounts in larvae exposed to both spray and oral treatments of both fungal isolates decreased with increasing conidial concentration. This situation may mean that GSH is insufficient to protect the larvae from the destructive effects of free radicals at high conidial concentrations. In a study in which *Periplaneta americana* (Linnaeus, 1758) was treated with different fungal isolates (Babu & Padmaja 2014), it was determined that the GSH amounts in insects differed with infection. GSH is upregulated in response to infection (Huang et al. 2011), implying that GSH plays a key role in detoxification. In our study, the opposite result was obtained. This result proves that free radicals are much more than GSH can eliminate at high conidial concentrations. In this study, the amounts of MDA and NOx were maximal in larvae exposed to the highest concentration of the ORP-18 isolate. In contrast, the GSH amount was minimal in this group. This indicates that GSH is insufficient especially in the face of ROS production by the larvae in this group. Moreover, it is not surprising that high ROS leads to the highest mortality in this group, as ROS underlies evolutionary tradeoffs between immunity and life-history traits such as survival (Zug & Hammerstein 2015).

Conclusion

The results showed that the *M. brunneum* ORP-18 isolate was more virulent than *B. bassiana* GOPT-331 isolate. In addition, it was found that with increasing conidial concentrations of both isolates, the amounts of MDA and NOx increased, while the GSH amount decreased. The low GSH amount indicates that it is not sufficient to overcome ROS and explains the decreased larval survival. The ORP-18 isolate can be recommended as a spray in biological control against *M. neustria* larvae. In field applications, these effects may differ. As a result, these fungal isolates should be tested under field under controlled conditions.

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