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Impact of *Beauveria bassiana* and *Metarhizium anisopliae* on biochemical and antioxidant enzymes in *Rhynchophorus ferrugineus* (Olivier) infesting oil palm

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ABSTRACT

Entomopathogenic fungi (EPF) are naturally occurring biological control agents that significantly manage pest populations in agriculture. It isagood, eco-friendly alternative to chemical pesticides. EPF's importance in pest control lies in its ability to target specific pests, reduce resistance development compared to synthetic chemicals, and minimise harmful environmental impacts. The present study was conducted to investigate the effectiveness of EPF against the pest of oil palm known as *Rhynchophorus ferrugineus*. For this study, the EPF was isolated from soil by using the insect baiting method. The isolated entomopathogenic fungi belonged to two genera, namely *Beauveria* and *Metarhizium*. The life cycle of the red palm weevil (RPW) was studied under laboratory conditions, and it took 140±26 days to complete one generation. After three generations of lifespan, the RPW grubs were treated with EPF in different concentrations of 2%, 4%, 6%, 8%, and 10%, respectively. Further, the infected samples and controls were analysed by biochemical parameters, antioxidant enzymes, and the results showed that these two *Beauveria bassiana* and *Metarhizium anisopliae* can control the activity of RPW. Thus, it can be used as a biopesticide in the future as an alternative source of other agrochemical pesticides.

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INTRODUCTION

The *Rhynchophorus ferrugineus,* also known as the red palm weevil (RPW),is a major destructive, deadly pest to *Coccus nucifera* (coconut*), Elaeis guineensis* (oil palm), *Euterpe edulis* (juçara), *Metroxylon sagu* (sago palm), *Phoenix canariensis* (pineapple palm), *Phoenix dactylifera* (date palm), and *Saccharum officinarum* (sugarcane). Palm species are very important economic crops throughout the world. One of the palm species known as oil palm is attacked by numerous insects globally, more than 193 species, including defoliators, leaf/fruit scrapers, borers, and sap feeders. Among these insects, RPW is a serious, devastating pest of the oil palm. In India, it was first discovered as a serious pest of the coconut palm (Lefroy, 1906). *Rhynchophorus ferrugineus* (olivier) was classified under the order Coleoptera, the family Curculionidae, and the subfamily Rhynchophorinae. It is a holometabolous insect which has 4 stages of life cycle, namely egg, larva, pupa, and adult.

Since it is a major pest of oil palm farms, friendly management techniques must be implemented. There are many biological methods to control this pest, and among those, entomopathogens are very effective.

Entomopathogens are microorganisms that can cause diseases in insects and are classified into various groups, such as protozoans, viruses, bacteria, fungi, and nematodes. In this, entomopathogenic fungi (EPF) is a group of fungi that live in soil that infects insects by penetrating their bodies through the cuticle of the pest and eventually kills them by feeding on them. This present study involves the survey and isolation of entomopathogenic fungi from soil. Also, isolated fungi were identified by preliminary level identification under the microscope, and the morphological characteristics show they belonged to *Beauveria bassiana* and *Metarhizium anisopliae.* The objective of the study is 1) to study the virulence of isolated fungi against RPW, 2) to study the efficacy of the entomopathogenic fungi and their mechanisms against RPW, and 3) to determine the activity against RPW through biochemical and antioxidant assays.

MATERIALS AND METHODS

Isolation of entomopathogenic fungi

Rearing of Galleria mellonella

For the isolation of EPF, the area was Semmedu (West), which is inthe Coimbatore district of Tamil Nadu. The *G. mellonella* (greater wax moth) larvae were used for baiting the EPF.

Hence, for the study, *G. mellonella* was cultured and maintained at room temperature till the end of the study. The life cycle of *G. melonella* was studied under laboratory conditions at 24 °C±2 °C. Males took 50±1 days; females took 51±1 days to complete one generation on an artificial diet.

Collection of soil samples

The soil samples were collected from cultivated coconut fields. The samples were taken randomly in the field, and more than 20 different spots were checked on the same land, but 12 soil samples were collected and stored at room temperature. Out of 12 samples, only 2 samples harboured EPF. At each zone, 250g of the soil samples were taken to a depth of 15-20cm by using the spade and crowbar and collected in clean polythene bags.

Insect baiting method

The baiting method, as described by Bedding and Akhurst (1975), involved placing each collected soil sample in a 500mg plastic container, which only contained 50g of soil samples,was baiting it with the last instar larva of *G. mellonella*. Ten larvae of *G. mellonella* were placed on top of each soil sample, and these containers were closed with khada cloth. The larvae were checked for infection every day, and death was observed within 16 hours, followed by 18 hours.

Genomic DNA isolation

DNA isolation from microbial samples was done using the EXpure Microbial DNA isolation kit developed by Bogar Bio Bee Stores Pvt Ltd.

Protocol

DNA was extracted by first lysing the samples in lysis buffer and RNAs, followed by incubation at 65 °C and

centrifugation to obtain a clear supernatant. The supernatant was then mixed with binding buffer and loaded onto a spin column in two steps. After allowing the DNA to bind, the column was washed sequentially with Washing Buffer I and Washing Buffer II, followed by a dry spin. The purified DNA was finally eluted by adding elution buffer to the centre of the column and centrifuging to collect the DNA in a fresh tube. The concentration of the extracted DNA was measured using a Qubit 3.0 fluorometer (Castresana, 2000; Dereeper *et al.,* 2008; Edgar, 2004; Talavera and Castresana, 2007; Edwards *et al.,* 1989).

PCR Protocol

PCR was performed using genomic DNA isolated from the samples. Each 25µL reaction mixture contained 5µL of template DNA, 1.5µL each of forward and reverse primers, 5µL of nuclease-free water, and 12µL of 2× Taq Master Mix (containing Taq DNA polymerase, 0.4mM dNTPs, 3.2mM $MgCl_2$, and 0.02% bromophenol blue).

Mass rearing of RPW

Different diets were prepared for the mass rearing of RPW. The mouth parts of the grub are not fully developed in its early stages (1^{st}–4^{th} instar). Grubs were therefore kept on a soft diet of banana and apple fruit in the proportion of one banana to half an apple per five grubs. Their mouth parts were fully developed when they reached the fifth instar, so they were moved to a sugarcane stem diet that weighed 50g per five grubs, based on the ratio they maintained.

Experimental analysis

For analysis, *B. bassiana* and *M. anisopliae*-infected RPWs and control grubs were homogenised with 2 mL of 5% trichloroacetic acid and centrifuged for twenty minutes at 10,000 rpm at 4°C.

Biochemical studies

Estimation of protein

Bradford (1976) method, bovine serum albumin was used as the reference to measure the protein content of control and infected RPWgrubs that were infected with two samples of EPF, namely *B. bassiana* and *M. anisopliae*. 500mg of the sample was homogenised in 2 mL of 8% trichloroacetic acid buffer solution and centrifuged. The supernatant was used for protein estimation. The optical density was read at 530nm using a spectrophotometer. The results were given in µg/ mL protein.

Estimation of carbohydrates

This method was carried out by using the method of Hedge and Hofreiter (1962). The carbohydrate content of control and infected RPW grubs was determined with two samples of EPF, namely *B. bassiana* and *M. anisopliae*, determined by using glucose as the standard. 500mg of the sample was homogenised in 2 mL of 8% trichloroacetic acid buffer solution and centrifuged. The supernatant was used for carbohydrate estimation. The optical density was read at 620nm using a spectrophotometer. The results were given in g carbohydrate µg/mL.

Estimation of lipids

Folch (1957) method, the lipid content of control and infected RPW grubs was determined with two samples of EPF, namely *B. bassiana* and *M. anisopliae*, determined by using olive oil as the standard. For the sampling process, the supernatant was left undisturbed for 10 minutes after 1 mL of a chloroform: methanol combination and 1 mL of 0.9% NaCl were added. 5 mL of 2% phosphovanillin was added to each test tube after 2 mL of concentrated sulfuric acid had been added, cooked for 20 minutes in a hot water bath, cooled, and left to stand for 10 minutes. To measure the absorbance at 540nm by using a UV-spectrophotometer.

Estimation of antioxidant enzyme activity

Catalase assay (CAT)

CAT assay activity was measured using the Wang *et al.* (2001) technique. The whole grub homogenate 500µl was mixed with 500µl of H_2O_2 and 2 mL of phosphate buffer, and the results were compared to a control cuvette containing only H_2O_2 and phosphate buffer. At 240nm, absorbance was measured.

The change in absorbance was recorded every 30 seconds up to 2 minutes in a spectrophotometer.

Superoxide dismutase (SOD)

SOD activity was measured using Marklund and Marklund's method (1974), The prepared reaction mixture was added to 100µl of grub homogenate extract and incubated at 30 °C for 5 minutes. At last, 80µl of 50µm riboflavin was added. The tubes were exposed to a 200W fluorescent lamp for 10 minutes. After exposure, 1mL of Griess reagent was added. The absorbance of the colour formed was measured at 543nm.

Peroxidase assay (POD)

POD activity was assessed by Reddy *et al.* (1995), 2400µl of pyrogallol solution and 100µl of the whole grub homogenate sample were mixed well with 0.5 mL of hydrogen peroxide. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer. Peroxidase was measured at 430nm with a UV–Visible spectrophotometer.

Ascorbate Peroxidase Assay (APX)

APX was assessed by Nakano and Asada (1981), 1 mL of sample was added to 2.7 mL of 100mm phosphate buffer solution (PBS) (pH 7.2), mixed well, then added with 100µl of L-ascorbic acid and 100µl of H_2O_2. At 290nm, absorbance was measured.

Polyphenol oxidase (PPO)

PPO was assessed by Gulen and Eris (2004), 300µl of enzyme extract was added to 1200µl of phosphate buffer, then mixed well, and after thorough mixing, 500µl of Catechol was added to adjust the final volume. A UV-visible spectrophotometer was used at 420nm to measure the absorbance.

RESULTS

Isolation of Entomopathogenic fungi from soil

The soil samples were collected from different spots on the same cultivated Coconut field. Out of 12 soil samples, two samples harboured EPF. Through the insect baiting method, the dead larvae of *G. mellonella* were separated from the soil and thoroughly washed with tap water, then placed in a petri dish and left for a week. After 7 days, the spores developed on the dead larvae's bodies were noticed and placed under the microscope to examine the spores' appearance.

Identification of entomopathogenic fungi

The samples were poured on PDA (Potato Dextrose Agar) media and left for 3 days. The morphological characteristics show that the isolated fungi belong to two genera, *Beauveria* and *Metarhizium*. Followed by the identification of the species, the genomic DNA was isolated, and it was also found to be *B. bassiana* and *M. anisopliae*. Both sp. of fungi were registered for NCBI submission.

The following sequences are the result of the samples

> Contig - GA_CO

GGGGATGGTTCGGGTTAACCTACCTGATTCGAGGT
CAACGTTCAGAAGTTGGGTGTTTTACGGCGTGGCC
GCGTCGGGGTCCCGGTGCGAGCTGTATTACTGCGC
AGAGGTCGCCGCGGACGGGCCGCCACTACCATTCT
CAGGGGACCGCGCGGATGTGCTAGCCGGTACCCCA
ACGCCGCACCTCCCCAAGGGGAGGTTGAGGTCTGG
AAATGACTGTCGAACAGGCATGCCCGCCATAATGC
TGGCGAATTGAATAATCCAGTGAATATTCGAATCTT
TGATGGCTCCTTGCGTCCGCCATAATTCTGGCGGTT
ATGTCTGTTCTAGCGTCATTTCAACCCTCGACCTCC
CCTTGGGGAGGACGTTGATTGGTTACCGTTTACCT
CGCCGCCGTATCCTGAAAAGGATGGCAAGACCTGA
CCTTGAGGACCCCGGCGAAGCAATTGGTCTCGTAC
CGGAACCCCGAGGCCCCCACGCCGAAAAACCACCC
AACTTAAGGTCTCTAACCTGGAATGGGAGATGACA
ACCCCGGTGAATGATCCCTCATCAAGAACCCCTACG
AATGGATCATTACCGAGTTTTCAACTCCCTAACCCT
TCTGTGAACCTACCTATCGTTGCTTCGGCGGACTCG
CCCCAGCCCGGGACGCGGACTGGGACCAGCGGCCC
GCCGGGGACCTCAAACTCTTGTATTCCAGCATCTTC
TGAAATACGCCGCAAGGCAAAACAAATGAATCAAA
ACTTTTCAACAACGGAATCTCTTGGGCTCTGGCATC
GATGAAGAACGCAGCGAAAACGCGATAAGTAATGG
TGAAATTTGCAGAATCCAGTGAAAATCATCGAATCT
TTGAACGCACATTGCGCCCGCCAGCATTCTGGCGG
GCATGCCTGTTCGAGCGTCATTTCAACCCTCGACCT
CCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCA

CACCGCCGGCCCTGAAATGGAGTGGCGGCCCGTCC
GCGGCGACCTCTGCGCAGTAATACAGCTCGCACCG
GGACCCCGACGCGGCCACGCCGTAAAACACCCAACT
TCTGAACGTTGACCTCGAATCAGGTAGGACTACCCGC
TGAACTTAAGCATATCAATAAGCCGGAGGAAGAT

B. bassiana

> Contig - GA_CO2

CATCCTGGGGTACCTACCTGATTCGAGGTCAACTAT
AAAAAGTTGGGGGGTTTTACGGCAGTGGACCGCGC
CGGGCTCCTGTTGCGAGTGTTTTACTACTGCGCAG
AGGAGGGCCACGGCGAGACCGCCAATTAATTTAAG
GGACGGCTGTGCTGGAAAACCAGCCTCGCCGATCC
CCAACACCAAGTCCACAGGGGACTTGAGGGGCGTA
ATGACGCTCGAACAGGCATGCCCGCCAGAATACTG
ACGGGCGCAATGTGCGTTCAAAGATTCGATGATTC
ACTGAATTCTGCAATTCACATTACTTATCGCATTTC
GCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCC
GTTGTTGAAAGTTTTGATTCATTTTTTTTAACCACT
CAGAAGATACTTATTAAAAAATTCAGAAGGTTTGG
GTCCCCGGCGGGCGCGAAGTCCCGCCGAAGCAACA
ATTAAAGGTATAATTCACAGGGGTTGGGAGTTGGA
TAACTCGGTAATGATCCCTCCGCAGGTTCACCTACG
GAAAAGGATCATTACCGAGTTATCCAACTCCCAACC
CCTGTGAATTATACCTTTAATTGTTGCTTCGGCGGG
ACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATT
TTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAAAT
GAATCAAAACTTTCAACAACGGATCTCTTTGGTTCT
GGCATCGATGAAGAACGCAGCGAAATGCGATAAGT
AATGTGAATTGCAGAAATTCAGTGAATTCATCGAAT
CTTTGAACGCACATTGCGCCCGTCAGTATTCTGGCG
GGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGT
CCCCTGTGGACTTGGTGTTGGGGGATCGGCGAGGC
TGGTTTTCCAGCACAGCCGTCCCTTAAATTAATTGG
CGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAA
AACACTCGCAACAGGAGCCCGGCGCGGTCCACTGC
CGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAA
TCAGGTAGGACTACCCGCTGAACTTAAGCATATCAA
TAAGCCGGAGGAAAAG

M. anisopliae

B. bassiana and M. anisopliae infected RPW

RPW grubs were infected with different dosages of 2%, 4%, 6%, 8% and 10%, respectively. In a 10% dosage, the death of RPW grub was observed at 16 hours and 18 hours for both fungi. The infected grub, along with the control, was taken to further studies, like biochemical and antioxidant enzymatic activity.

Effect of EPF on grub mortality

The mortality of grubs increased progressively with increasing concentrations of the EPF- *B. bassiana* and *M. anisopliae* after four days of treatment. In the untreated control, mortality remained relatively low. Application of *B. bassiana* resulted in a gradual rise in mortality, reaching 50% at 6% concentration and 70% at 8%. The highest mortality (90%) was recorded at 10% concentration.

A similar development was observed for *M. anisopliae*. The control treatment exhibited comparatively lower mortality, whereas fungal treatments caused a substantial increase in grub mortality. Mortality reached 60% at 6% concentration and increased to 70% at 8%. The maximum mortality (90%) was also observed at 10% concentration (Table 1, Fig. 1).

Table 1. Mortality of grubs treated with different concentrations of EPF

Treatment	Concentration	Mean mortality (%) ± SD
Control	—	20.00 ± 0.00
B. bassiana	6%	50.00 ± 14.14
B. bassiana	8%	70.00 ± 14.14
B. bassiana	10%	90.00 ± 14.14
M. anisopliae	6%	60.00 ± 0.00
M. anisopliae	8%	70.00 ± 14.14
M. anisopliae	10%	90.00 ± 14.14

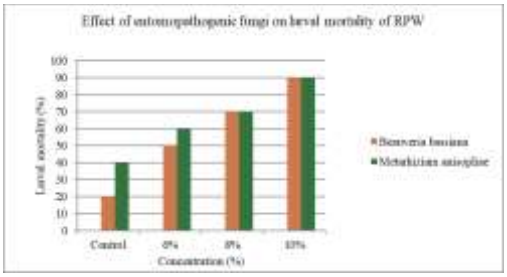

Fig. 1. Effect of different concentrations of EPF on grub mortality (%)

Biochemical composition of RPW grub

Protein content

Protein content in the grub showed significant differences among treatments with the EPF - *B.*

bassiana and *M. anisopliae*. The control group recorded the highest protein level (1.59 ± 0.089 µg mL^{-1}) for both fungi. In the grub treated with *B. bassiana*, protein content decreased with increasing concentration, with values of 1.30 ± 0.086 and 1.44 ± 0.068 µg mL^{-1} at 6% and 8%, respectively. The lowest protein content (0.79 ± 0.031 µg mL^{-1}) was observed at 10% concentration.

Similarly, in *M. anisopliae* treatments, protein levels were slightly reduced at 6% (1.53 ± 0.026 µg mL^{-1}) and 8% (1.50 ± 0.020 µg mL^{-1}), while a marked decline was observed at 10% concentration (0.83 ± 0.026 µg mL^{-1}).

Overall, higher fungal concentrations resulted in a reduction in protein content. These findings indicate that infection by *B. bassiana* and *M. anisopliae* leads to a depletion of protein reserves in the grub (Table 2, Fig. 2).

Table 2. Effect of different concentrations on protein content in the grub treated with the *B. bassiana* and *M. anisopliae*

Concentration	*B. bassiana* (Mean ± SD)	*M. anisopliae* (Mean ± SD)
Control	1.59 ± 0.089^{c}	1.59 ± 0.089^{c}
6%	1.30 ± 0.086^{b}	1.53 ± 0.026^{b}
8%	1.44 ± 0.068^{b}	1.50 ± 0.020^{b}
10%	0.79 ± 0.031^{a}	0.83 ± 0.026^{a}

Means followed by different superscript letters differ significantly at $p \leq 0.05$ according to Tukey HSD test

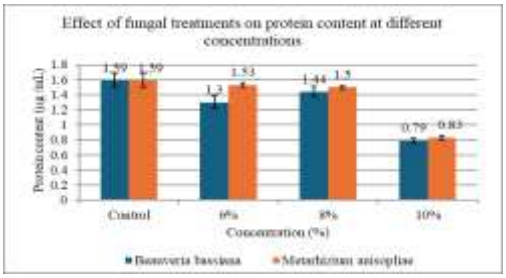

Fig. 2. Effect of the *B. bassiana* and *M. anisopliae* on protein content at different concentrations

Bars represent mean values and error bars indicate standard deviation (n = 3). Different superscript letters indicate significant differences at $p \leq 0.05$.

Carbohydrate content

Carbohydrate content in the grub varied among treatments with the EPF *B. bassiana* and *M. anisopliae*. The control group showed the highest carbohydrate level (1.45 ± 0.34 µg mL^{-1}).

In grub treated with *B. bassiana*, carbohydrate content decreased markedly, recording 0.37 ± 0.06, 0.70 ± 0.02, and 0.55 ± 0.26 µg mL^{-1} at 6%, 8%, and 10% concentrations, respectively.

A similar reduction was observed in *M. anisopliae* treatments. Carbohydrate levels were 0.42 ± 0.05 µg mL^{-1} at 6%, 0.63 ± 0.21 µg mL^{-1} at 8%, and 0.54 ± 0.01 µg mL^{-1} at 10%, all of which were lower than the control (Table 3, Fig. 3).

Table 3. Effect of different concentrations on carbohydrate content in the grub treated with *B. bassiana* and *M. anisopliae*

Concentration	*B. bassiana* (Mean ± SD)	*M. anisopliae* (Mean ± SD)
Control	1.45 ± 0.34^{b}	1.45 ± 0.34^{b}
6%	0.37 ± 0.06^{a}	0.42 ± 0.05^{a}
8%	0.70 ± 0.02^{a}	0.63 ± 0.21^{a}
10%	0.55 ± 0.26^{a}	0.54 ± 0.01^{a}

Means followed by different superscript letters differ significantly at $p \leq 0.05$ according to Tukey HSD test

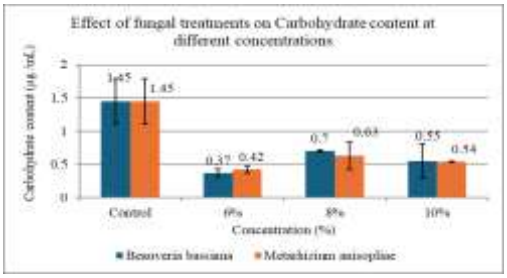

Fig. 3. Effect of the *B. bassiana* and *M. anisopliae* on carbohydrate content at different concentrations

Bars represent mean values and error bars indicate standard deviation (n = 3). Different superscript letters indicate significant differences at $p \leq 0.05$.

Lipid content

Lipid content in the grub decreased noticeably following treatment with the lipid content of grubs treated with EPF showed a noticeable reduction compared with the control group. In the *B. bassiana* treatment, the control recorded the highest lipid content (1.40 ± 0.31), whereas the lowest lipid level was observed at 6% concentration (0.35 ± 0.03). The

8% concentration showed a moderate lipid level (0.61 ± 0.04), while the 10% concentration resulted in a reduced lipid content (0.39 ± 0.05).

Similarly, in the *M. anisopliae* treatment, the control group exhibited the highest lipid content (1.40 ± 0.31). Among the treated groups, the highest lipid content was observed at 8% concentration (0.82 ± 0.02), followed by 10% (0.62 ± 0.04) and 6% (0.60 ± 0.02). Overall, fungal treatments resulted in a significant reduction in lipid content compared to the untreated control (Table 4, Fig. 4).

Table 4. Effect of different concentrations on lipid content in the grub treated with *B. bassiana* and *M. anisopliae*

Concentration	*B. bassiana* (Mean ± SD)	*M. anisopliae* (Mean ± SD)
Control	1.403 ± 0.306^{b}	1.403 ± 0.306^{b}
6%	0.347 ± 0.031^{a}	0.600 ± 0.020^{a}
8%	0.610 ± 0.036^{a}	0.817 ± 0.015^{a}
10%	0.387 ± 0.047^{a}	0.617 ± 0.035^{a}

Different superscript letters indicate significant difference at $p \leq 0.05$ (Tukey test).

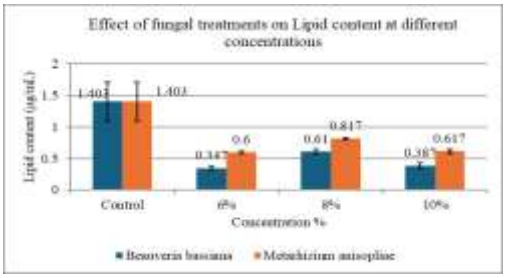

Fig. 4. Effect of different concentrations of *B. bassiana* and *M. anisopliae* on lipid content (µg/mL) of grubs

Bars represent mean values and error bars indicate standard deviation (n = 3). Differences at $p \leq 0.05$ according to Tukey's HSD test.

Antioxidant enzyme activity

CAT

CAT activity showed slight variation among the different treatments. In *B. bassiana*, the highest CAT activity was recorded in the control treatment (1.18 µg/mL). The treated concentrations exhibited comparatively lower values, with 6% and 8% both recording 1.07 µg/mL and 10% showing 1.06 µg/mL. A comparable pattern was observed in *M. anisopliae*, where the control treatment also registered the maximum catalase activity (1.18 µg/mL), while 6%, 8%, and 10% concentrations showed reduced activities of 1.06 µg/mL (Table 5, Fig. 5).

Table 5. Effect of different concentrations on SOD activity in the grub treated with *B. bassiana* and *M. anisopliae*

Concentration	*B. bassiana* (Mean ± SD)	*M. anisopliae* (Mean ± SD)
Control	0.226 ± 0.003^{d}	0.226 ± 0.003^{d}
6%	0.075 ± 0.002^{a}	0.079 ± 0.003^{a}
8%	0.120 ± 0.003^{c}	0.106 ± 0.000^{c}
10%	0.226 ± 0.003^{d}	0.226 ± 0.003^{d}

Means followed by different superscript letters indicate significant differences at $p \leq 0.05$ according to Tukey's HSD test.

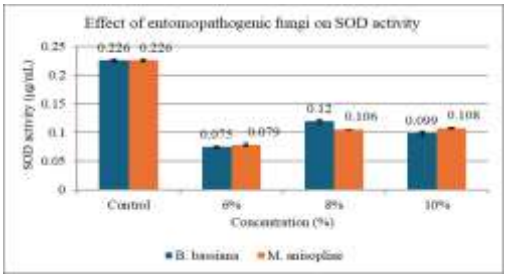

Fig. 5. Effect of different concentrations of *B. bassiana* and *M. anisopliae* on SOD activity (µg/mL)

Values represent mean ± SD (n = 3). Different superscript letters indicate significant differences among treatments according to Tukey HSD test ($p \leq 0.05$).

SOD

In *B. bassiana*, the control treatment showed the highest SOD activity (0.226 µg/mL). Among the treated concentrations, 8% recorded relatively higher activity (0.120 µg/mL) followed by 10% (0.099 µg/mL), while the minimum activity was observed at 6% concentration (0.075 µg/mL). A similar trend was noticed in *M. anisopliae*, where the control treatment also exhibited the highest SOD activity (0.226 µg/mL). The treated concentrations showed reduced activity, with 10% (0.108 µg/mL) and 8% (0.106 µg/mL) recording intermediate values and the lowest activity observed at 6% concentration (0.079 µg/mL) (Table 6, Fig. 6).

Table 6. Effect of different concentrations on CAT activity in the grub treated with the *B. bassiana* and *M. anisopliae*

Concentration	*B. bassiana* (Mean ± SD)	*M. anisopliae* (Mean ± SD)
Control	1.181 ± 0.002[b]	1.181 ± 0.002[b]
6%	1.073 ± 0.002[a]	1.056 ± 0.003[a]
8%	1.068 ± 0.004[a]	1.065 ± 0.002[a]
10%	1.061 ± 0.007[a]	1.060 ± 0.001[a]

Means followed by different superscript letters indicate significant differences at $p \leq 0.05$ according to Tukey's HSD test

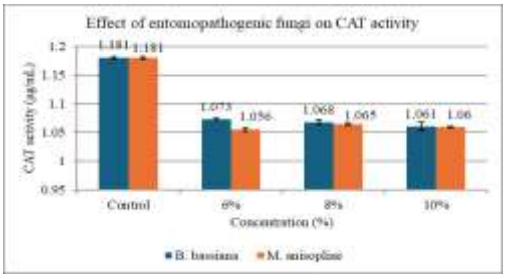

Fig. 6. Effect of different concentrations of *B. bassiana* and *M. anisopliae* on CAT activity (µg/mL)

Values represent mean ± SD (n = 3). Different superscript letters indicate significant differences among treatments according to Tukey HSD test ($p \leq 0.05$).

POX

In *B. bassiana*, the control treatment exhibited the highest activity (1.15 µg/mL), whereas all treated concentrations displayed lower values. The activities recorded were 0.76 µg/mL at 6%, 0.75 µg/mL at 8%, and 0.77 µg/mL at 10%. Similarly, in *M. anisopliae*, the control treatment showed the maximum activity (1.15 µg/mL), while the treated concentrations (6%, 8%, and 10%) recorded similar and comparatively lower values of around 0.76 µg/mL (Table 7, Fig. 7).

Table 7. Effect of different concentrations on POX activity in the grub treated with *B. bassiana* and *M. anisopliae*

Concentration	*B. bassiana* (Mean ± SD)	*M. anisopliae* (Mean ± SD)
Control	1.181 ± 0.002[b]	1.181 ± 0.002[b]
6%	1.073 ± 0.002[a]	1.056 ± 0.003[a]
8%	1.068 ± 0.004[a]	1.065 ± 0.002[a]
10%	1.061 ± 0.007[a]	1.060 ± 0.001[a]

Means followed by different superscript letters indicate significant differences at $p \leq 0.05$ according to Tukey's HSD test

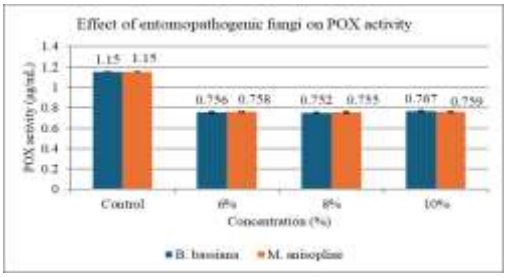

Fig. 7. Effect of different concentrations of *B. bassiana* and *M. anisopliae* on POX activity (µg/mL)

Values represent mean ± SD (n = 3)

Means followed by the same superscript letters indicate no significant differences among treatments according to Tukey HSD test ($p \leq 0.05$).

APX

In *B. bassiana*, the highest APX activity was observed in the control treatment (2.209 µg/mL). Among the treated concentrations, 8% showed relatively higher activity (2.034 µg/mL), followed by 6% (1.999 µg/mL), while the lowest value was recorded at 10% concentration (1.634 µg/mL) (Table 8, Fig. 8).

Table 8. Effect of different concentrations on APX activity in the grub treated *B. bassiana* and *M. anisopliae*

Concentration	*B. bassiana* (Mean ± SD)	*M. anisopliae* (Mean ± SD)
Control	2.209 ± 0.010[c]	2.209 ± 0.010[c]
6%	1.999 ± 0.003[b]	2.018 ± 0.002[b]
8%	2.034 ± 0.048[b]	1.941 ± 0.003[b]
10%	1.634 ± 0.001[a]	1.776 ± 0.002[a]

Means followed by different superscript letters indicate significant differences at $p \leq 0.05$ according to Tukey's HSD test

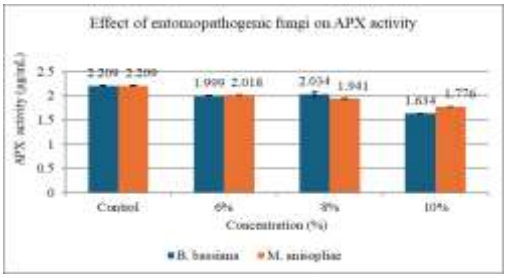

Fig. 8. Effect of different concentrations of *B. bassiana* and *M. anisopliae* on APX (µg/mL)

Values represent mean ± SD (n = 3). Different superscript letters indicate significant differences among treatments according to Tukey HSD test ($p \leq 0.05$).

In *M. anisopliae*, the control treatment similarly recorded the highest APX activity (2.209 µg/mL). The treated concentrations showed slightly lower activities, with 6% (2.018 µg/mL) and 8% (1.941 µg/mL), whereas the lowest activity was observed at 10% concentration (1.776 µg/mL).

Ppo

In *B. bassiana*, the control treatment recorded the highest activity (1.13 µg/mL). In contrast, the treated concentrations showed markedly lower activities, with 6% (0.125 µg/mL), 10% (0.103 µg/mL), and 8% (0.095 µg/mL). Likewise, in *M. anisopliae*, the control treatment also showed the maximum PPO activity (1.13 µg/mL), whereas the treated concentrations displayed reduced values of 0.114 µg/mL at 6% and 10%, and 0.135 µg/mL at 8% (Table 9, Fig. 9).

Table 9. Effect of different concentrations on PPO activity in the grub treated with *B. bassiana* and *M. anisopliae*

Concentration	*B. bassiana* (Mean ± SD)	*M. anisopliae* (Mean ± SD)
Control	2.209 ± 0.010^{c}	2.209 ± 0.010^{c}
6%	1.999 ± 0.003^{b}	2.018 ± 0.002^{b}
8%	2.034 ± 0.048^{b}	1.941 ± 0.003^{b}
10%	1.634 ± 0.001^{a}	1.776 ± 0.002^{a}

Means followed by different superscript letters indicate significant differences at $p \leq 0.05$ according to Tukey's HSD test.

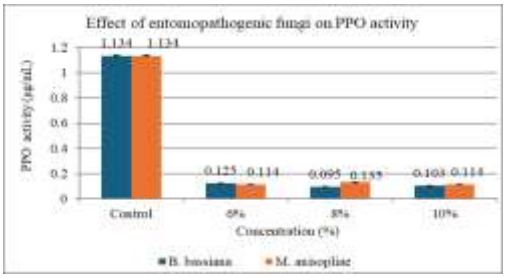

Fig. 9. Effect of different concentrations of *B. bassiana* and *M. anisopliae* on PPO activity (µg/mL) Values represent mean ± SD (n = 3). Different superscript letters indicate significant differences among treatments according to Tukey HSD test ($p \leq 0.05$).

DISCUSSION

Green biocontrol is effective against the target insect, decomposes quickly, and maintains a high and safe crop output without harming the ecosystem, it is a viable solution to the RPW infestation. Numerous RPW-related natural antagonists, including viruses (*Baculovirusoryctes, Cytoplasmic polyhedrosisvir*us), bacteria (*Bacillus* sp., *Pseudomonas aeruginosa),* fungi (*Metarhizium anisopliae*, *Beauveria bassiana*), and nematodes (*Heterorhabditis indica*, *Steinernema abbasi*), can be important pest biocontrol agents. The EPF is particularly well-suited since direct contact with the insect cuticle might result in infection by a combination of enzymatic breakdown and mechanical pressure without swallowing. Especially, the forest soil and agricultural soil naturally contain the EPF.In several studies, the use of EPF as a microbial agent has been able to control the pest RPW.

The isolation of EPF from the soil involves different types of methods, in which the insect baiting method is best for isolating EPF (Dana *et al.,* 2023).

Sutanto *et al.* (2021) studyresults showed that within three to seven days, *B. bassiana* and *M. anisopliae* isolates with the concentration 1×1010 conidia/ mL showed an 83% to 100% mortality rate. Local isolates have shown high virulence in contrast to the exotic ones. This can be a promising alternative source to a commercial one. Here, in this present study,the isolated two sp. of fungi, namely *B. bassiana* and *M. anisopliae,* killed the pest within 16 hours, followed by 18 hours, which can control the pest quickly compared to other insecticides.

In the present study, theproteins, carbohydrates, and lipids present in insect body fat and haemolymph are particularly interesting since they give us a sufficient background to examine the synthetic activities linked to the many stages in developing organisms.

According to the study of Abdel-Razek *et al.* (2004), protein concentration in the haemolymph of *Pectinophora gossypiella* was higher than the insect body fat. The result of this study was that the concentration of protein in the control was higher than in the infected fungi, *B. bassaina* and *M.*

anisophilae. Protein inhibition or reduction appears to be a better explanation for various other experimental findings, including weight loss, delayed development, tissue deterioration, and delayed adult emergence. Here, this study examines the amount of protein reduced because of their growth and development.

Abdel-Razek *et al.* (2004) study revealed thatthe nematode-bacterial complex-treated *R. ferrugineus* total body fat had higher carbohydrate content than the control due to the physiological differences. In the control grub, the amount of carbohydrates in the haemolymph was assessed to be lower than in both treatments, but the amount of carbohydrates in the control grub was higher than in both treatments. Accordingly, the control group in this study had more carbohydrates than the infected group. Utilisation of reserves of stored carbohydrates may causea decrease in the amount of carbohydrates in the fat body of the treated grub. Moreover, proteins may be synthesised with the help of carbohydrates.

According to Abdel-Razek *et al.* (2004), the total lipid content in the haemolymph of *Spodoptera littoralis* and *Popillia japonica* larvae decreased when infected with *Bacillus thuringiensis* and *Bacillus popilliae* compared to the control. This was because the infected larvae may have produced enzymes that use the lipids from the haemolymph to try and eliminate the invasive organisms. These results are consistent with his total haemolymph lipid readings. The same observation has been noted in this present investigation because the present study control grub lipid was higher than the infected sp. of fungi.

The low protein, carbohydrate, and lipid content in the infected may be used by *B. bassiana* and *M. anisopliae* for their growth, development, and reproduction. The pathogens have also destroyed the hosts for RPW and the immune system for their development. These changes in protein, lipid, and carbohydrates are key aspects in different changes in the physiological actions of the pest. This could be of great value in the control of this serious pest in the agricultural field.

The present experiment is devised to understand the biochemical parameters like protein, lipid, carbohydrate and the antioxidant defence system induced at different dosages of infection with *B. bassiana* and *M. anisopliae* in the red palm weevil. The antioxidant enzymes like catalase, superoxide dismutase, peroxidase, ascorbate peroxidase and polyphenol oxidase, in all the infected compared to control, *B. bassiana* and *M. anisopliae,* had mycotoxins which had insecticidal activity and caused tetanic paralysis, it also hinders DNA, RNA, and protein synthesis, it targets the insect's innate immune signalling pathway. It also damages cellular components and impairs insect physiology.

CONCLUSION

This study lies in demonstrating that local isolates of *B. bassiana* and *M. anisopliae* induce exceptionally rapid mortality (within 16 to 18 hours) in RPW, far faster than previously reported EPF strains. By combining biochemical analyses with antioxidant enzyme profiling, the study provides the first integrated evidence that these EPF trigger severe oxidative stress, suppress immune signalling, and rapidly deplete essential macromolecules such as proteins, lipids, and carbohydrates, which are required for host growth and development. This mechanistic linkage between metabolic disruption, oxidative imbalance, and fungal virulence presents a significant advancement in understanding EPFhost interactions and establishes these local isolates as highly promising candidates for rapid, eco-friendly management of the red palm weevil.

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