# The Efficacy of Entomopathogenic Fungi for Antestia Bugs (*Antetiopsis intircata*: Pentatomidea, Hemimptera) Control

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**Abstract:** Antestia bug (*Antestiopsis intricata*) is the major coffee insect pest affecting coffee productions in Ethiopia. Therefore, the study was carried out to evaluate entomopathogenic fungi based management options for antestia bug control. The efficacy test of entomopathogenic fungi was done under laboratory condition in Jimma Agricultural Research Center, Entomology and Pathology laboratories. The entomopathogenic fungi isolates were brought from Haramaya University and Ambo Agricultural Research Center. Seven isolates of *Beauvaria bassiana* (DLCO-12, APPRC-0247, DLCO-90, DLCO-56, DLCO-76, PPRC-44BC, and PPRC-27J) and four isolates of *Metarhizium ansopliae* (PPRC-51, DLCO23A, DLCO-91 and PPRC-02BC) species were tested for germination. Completely Randomized Design with three replications and probit analysis were used for data analysis by using SAS, GenStat and Microsoft excel. Eight isolates: APPRC 0247, DLCO 90, DLCO 56, DLCO 76, PPRC-44BC, PPRC-27J, PPRC-51 and PPRC-02BC which recorded higher germination were used for pathogenicity test against antestia bugs. All the tested isolates killed antestia bugs but their levels of efficacy differ across exposure time. Among tested isolates, PPRC-44BC and PPRC-27J registered complete mortality against antestia bugs and lower median lethal time, $LT_{50}$ (9.98 and 10.98 days, respectively). As a result these were taken as promising isolates and used for further isolates rate test. PPRC-44BC and PPRC-27J isolates applied at $1x10^8$ conidia ml$^{-1}$ killed all the tested antestia bugs in shorter exposure time. Even though PPRC-44BC and PPRC-27J isolates showed promising results under laboratory conditions, it needs further investigations on its formulation and under field conditions. Isolations of biocontrols from natural coffee growing areas and the effect of biocontrols against natural enemies should be studied.

**Keywords:** *Coffea arabica*, Pathogenicity, Rate, bio pesticides, PPRC-27J, PPRC-44BC

## 1. Introduction

Arabica coffee (*Coffea arabica* L.) was originated in the Afromontane rainforests highlands of southwest and southeast Ethiopia where wild coffee populations naturally occur [1]. It is the only coffee species grown in Ethiopia, and it plays a significant role in Ethiopian economy [2]. Arabica coffee is the most widely consumed, dominating over 70% in volume of production and over 90% of traded value globally [3]. Coffee shared 4.94% of the area under all crops in the Ethiopia and 4,690,911.24 quintals of produce was obtained

from this crop in the 2016/2017 production year [4]. The contributions of coffee to Ethiopia's economy is more than 25 - 30% of the country's foreign exchange earnings, 60% of the GDP, 12% of the agricultural sector output, and 20% of the government revenues [5].

Although, coffee plays a significant role in the economy of Ethiopia, the crop suffers from many production constraints [6-8]. Among the major factors limiting coffee productions are losses due to pests (insects, disease, nematodes and weeds) estimated to be 13%. But, in Africa the yield losses can be higher up to 96% [9]. The increasing incidences of coffee pests and their consequent control and management have

significantly constrained economical production of coffee in Kenya [10]. Liebig reported that coffee production worldwide is threatened by a range of coffee pests [11]. The most serious insect pests can cause coffee farmers to lose up to 20% of the crop and reduce the coffee value by 30 to 40% [12]. Additionally, they indicated that the international marketing policies do not allow coffee for export if more than 1.5% bean damage caused by insect pests.

Antestia bug is the major one inflicting considerable damage from identified coffee insect pests of Ethiopia [13]. *Antestiopsis intricata* (Ghesquiere and Carayon) is the most common antestia bug found in all coffee growing areas of Ethiopia except Hararghe, where only *Antestiopsis facetoides* is found [13]. The adults and nymphs are the damaging stages and they feed mostly on immature green berries, from which they suck the sap, causing the fruits to shrink [14]. They can feed on shoots and leaves of coffee plants but prefer to attack unripe coffee cherries [15]. Antestia bug also vectors pathogens causing infection of coffee cherries with bacteria and fungi by drilling a small hole into the skin of the coffee fruit [16-18]. In Ethiopia, antestia bug causes 9% yield loss and 48% coffee bean darkening. The presence of 2-3 antestia bugs per tree in the field can cause about 45% crop loss [19]. In Burundi and Rwanda it can cause loss up to 30% if not controlled and affect the quality of coffee as well [20].

Biopesticides based on microbial control agents are one of the potential options for insect pest management and development of integrated pest management (IPM) of modern agricultural practices. The use of biopesticides based entomopathogenic fungi (EPF) are among the least considered and insufficient information is available for coffee pest management especially in Ethiopia. Magina *et al.* reported that there is a serious interest in the use of microbial insecticides for biological control of insect pests, as alternatives to chemical control [21-23]. They neither leave toxic chemical residues in the environment nor do they induce resistance in their insect hosts. The public awareness and concern for environmental quality, has led to more focused attention on research aiming at developing biological agents. In addition, EPF have an essential role in IPM if they can be used in conjunction with other strategies for sustainable pest control [24]. Therefore, this study was initiated with the objectives to evaluate the efficacy of different EPF isolates of *Beauvaria bassiana* and *Mettarhizium ansopliae* for antestia bugs control.

# 2. Materials and Methods

## 2.1. Description of the Study Area

The study was conducted in southwestern Ethiopia, Jimma Agricultural Research Centre (JARC), Entomology and Pathology laboratories. It is situated within the Tepid to cool humid highlands agro-ecological zone of the country located at around 7° 46' N latitude and 36° E longitude and at an elevation of 1750 meters above sea level [25]. The site receives high amount of rainfall with a long-term mean total of 1573.6 mm per annum, which is distributed into 166 days.

The driest months usually last between November and February. The mean maximum and minimum air temperatures are 26.3 and 11.6°C, respectively [26].

## 2.2. Antestia Bugs Rearing

Antestia bug rearing was carried out in Entomology laboratory of Jimma Agricultural Research Center (JARC). Antestia bug was collected from coffee plantations of Jimma and Mettu area. After sufficient bugs were collected, for rearing purpose, the collected bugs were put in appropriate containers, and green coffee berries was used to feed them. And they were maintained in cool temperature and shaded area and brought into JARC, Entomology laboratory. Antestia bugs were reared in a glass cage covered with nylon mesh as indicated by Esayas *et al.* [27].

Fresh coffee twigs bearing green berries were provided for the insect at 2-3 days intervals [28]. Although the antestia bug feeds on various parts of the coffee plant, it has a strong preference for mature green berries which are essential for the bug to complete its life cycle, reproduce and enhance its longevity (Figure. 1b) [29]. The combined coffee leaves and coffee green berries were used as rearing substrate. Coffee leaves may improve relative humidity in the containers or provide nymphs with hiding places and helping them to escape from intense light in incubators and the complete life cycle was obtained [30].



*Figure 1. Antestia bugs rearing in laboratory a) Antastia bugs rearing cages and b) Green berry with leaf to feed antestia bugs.*

## 2.3. Preparation of Fungal Isolates

Five of EPF isolates (APPRC 0247, PPRC-44BC, PPRC-27J, PPRC-51 and PPRC-02BC) were procured from Ambo agricultural research center and six were from (DLCO 12, DLCO 90, DLCO 56, DLCO 76, DLCO23A and DLCO 91) Haramaya University, Entomology postgraduate laboratory. The isolates were obtained from the collection maintained by the Ethiopian Institute of Agricultural Research (EIAR) at the Ambo Agricultural Research Center and Desert Locust Control Organization for East Africa (DLCOEA) (Table 1). Four and seven *M. anisopliae* and *B. bassiana* isolates were used, respectively. Upon receipt, conidia were stored in dark condition at 4°C until used. The initial cultures of all isolates were stored at -5°C and sub-culturing was made as appropriate. For sub-culture, Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) by the production of aerial spores for the subsequent

laboratory bioassays were used. Two to three weeks old cultures were harvested in 10 ml of sterile distilled water containing 0.05% Tween-80% for use in the laboratory bioassays.
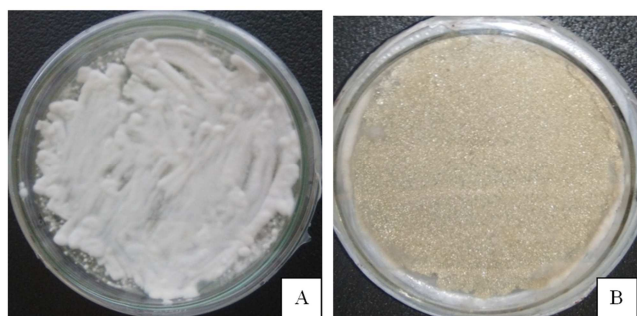


*Figure 2. Sporulating cultures of entomopathogenic fungi used in the screening experiment A) B. bassiana and B) M. anisopliae.*

## 2.4. Experimental Design and Treatments

Eleven EPF isolates with controls replicated thrice and laid out in Completely Randomized Design (CRD) were used (Table 1). In this experiment, there were controls (the untreated, distilled and sterilized water and Fenitrothion insecticide) for comparison and conducted under laboratory conditions. Five days old adult antestia bugs and five adults per petri dish were used for the experiment.

*Table 1. List of entomopathogenic fungi isolates which used for the study.*

| Fungal species | Fungal Isolate | Source |
|---|---|---|
| Beauveria bassiana | DLCO 12 | DLCOEA |
| ” | APPRC 0247 | AmARC |
| ” | DLCO 90 | DLCOEA |
| ” | DLCO 56 | DLCOEA |
| ” | DLCO 76 | DLCOEA |
| ” | PPRC-44BC | AmARC |
| ” | PPRC-27J | AmARC |
| Metarhizium anisopliae | PPRC-51 | AmARC |
| ” | DLCO23A | DLCOEA |
| ” | DLCO 91 | DLCOEA |
| ” | PPRC-02BC | AmARC |

DLCOEA: Desert Locust Control Organization for East Africa, AmARC: Ambo Agricultural Research Center

## 2.5. Fungal Isolates Viability Tests

Initially, eleven EPF isolates were evaluated for germination test. The conidial suspension was spread evenly on the agar medium with a sterile glass rod and each petri dish containing conidial culture was sealed with parafilm and incubated at 26±2°C for 20 to 24 hours and observed for germ tube development. Three plates were inoculated per sample batch of conidia and control was run concurrently with suspension oil only. These tests were carried out under sterile conditions inside a biohazard safety cabinet. After incubation, conidial viability was determined by quantifying spore germination (the formation of the distinct germ tube) using phase contrast microscopes at 400X magnification, fitted with a Neubaeur haematocytometer for spore counts [31]. Conidia were deemed to have germinated if germination tubes are longer than half the size of the conidia. Approximately 100 conidia/ plate were counted and the average of germinated versus non-germinated spores calculated in order to determine the percentage germination:

$$\% \text{ Germination } = \frac{(\text{Total germinated})}{(\text{Total number of spores counted})} \text{ X } 100$$

## 2.6. Pathogenicity of EPF Isolates Against Antestia Bugs

Eight EPF isolates (two isolates of *M. anisopliae* and six of *B. bassiana*) which showed higher germination were used for pathogenicity test. Five day old adult antestia bugs were transferred from rearing cage into container and covered with nylon mesh. These containers were transparent in order to observe the antestia without opening and perforated in order to permit air exchange for survival of the bugs. The green berries were put in each container to provide food to the antestia bugs. Insects were allowed to settle on the host plants (coffee green berry) and then sprayed with 10 ml/ container of aqueous suspension of each fungal isolate at a concentration of $1 \times 10^8$ conidia $ml^{-1}$ (standard infective concentration of $1.0 \times 10^8$ conidia $ml^{-1}$ in 0.05% Tween-80) and with controls using syringe. The suspensions were sprayed in equal quantity in each box containing these bugs and uniform distribution of the bio pesticide on the insect was ensured. The coffee berries were sprayed, too as described by Nahayo and Bayisenge [32].

The control groups were sprayed with sterile distilled water with 0.05% Tween-80 and Fenitrothion. Both control and treated insects were transferred to container (20 cm x 20 cm x 10 cm) lined with moistened filter paper and incubated at 26±2°C. All the fungal isolates were bio assayed concurrently against the antestia bug and replicated three times. Treatments were arranged in a completely randomized design. Five antestia bug adult per plate/container were used and mortality was recorded daily after application over exposure time. The dead insects were surface sterilized with 70% alcohol and then rinsed thrice in sterile distilled water and was kept separately in a petri dish lined with sterile moistened filter paper to initiate mycosis. And it was confirmed using a dissecting microscope.

## 2.7. Dosage Mortality Response Against Antestia Bugs

The promising fungal isolates from the screening tests (PPRC-27J and PPRC-44BC isolates) were tested for dosage response mortality assays at $1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$ and $1 \times 10^8$ conidia $ml^{-1}$ in laboratory conditions. The conidial suspensions were prepared by mixing the suspension using a magnetic stirrer for 5 min and the suspensions filtered through sterile muslin cloth to eliminate the coagulated medium. The spore suspensions were prepared for each isolates by using haemocytometer as indicated by Lacey and different concentrations were obtained through serial dilutions [33]. The control groups were sprayed with sterile distilled water containing 0.05% Tween-80 and Fenitrothion. All the fungal isolates were bio assayed concurrently against the antestia bug and replicated three times and treatments arranged in a RCD. Five antestia bug adults per plate/container were used and

mortality was recorded daily after treatment application and similar procedures in section 2.6 were followed.

### 2.8. Median Lethal Concentration (LC$_{50}$) and Time (LT$_{50}$)

Median lethal time (LT$_{50}$) and median lethal concentration (LC$_{50}$) were determined for each concentration by taking into account the time and concentration required at which the inocula of fungus caused 50% of the mortality on adult antestia bug population. LT$_{50}$ and LC$_{50}$ required to achieve 50% mortality per replicate were obtained from Probit analysis.

### 2.9. Data Analysis

Insect mortality, pathogenicity of fungal isolates and germination percentage of fungal isolates data were tested for normality using SAS software proc univariate. Where data violated the assumption of ANOVA, transformation of the data were done using square root transformation before running the analysis of variance and analyzed using the SAS version 9.3 and GenStat version-16. Tukey's test at 5% level

was used to compare treatment means [34, 35]. For LT$_{50}$ and LC$_{50}$, probit analysis was done using Polo Plus (LeOra Software) for each isolates at different concentrations.

## 3. Results

### 3.1. Viability of Entomopathogenic Fungi Isolates

The germination percentage of the tested entomopathogenic fungi isolates showed significant difference (p<0.05) (Table 2). The maximum mean germination percentage recorded was 93.00 from isolate DELCO-76 followed by isolate DELCO-90 with 90.33. On the other hand, minimum mean germination percentage recorded was 55.33 by isolate DELCO-91 followed by isolates DELCO-12 and DELCO-23A with 70.67 and 73.00, respectively. From eleven tested isolates, three isolates DELCO-91, DELCO-12 and DELCO-23A which showed lower mean germination percentages were discarded from further pathogenicity tests.

*Table 2. Percentage germinations of fungal spores on SDA plates after 18 h at 26±2°C.*

| No. | Fungal Isolate | Fungal species | % Germination (mean±SE) |
|---|---|---|---|
| 1 | DLCO 12 | *Beauveria bassiana* | 70.67[c]±1.453 |
| 2 | APPRC 0247 | " | 83.67[b]±0.667 |
| 3 | DLCO 90 | " | 90.33[ab]±0.882 |
| 4 | DLCO 56 | " | 90.00[ab]±1.155 |
| 5 | DLCO 76 | " | 93.00[a]±1.528 |
| 6 | PPRC-44BC | " | 89.67[ab]±0.882 |
| 7 | PPRC-27J | " | 89.00[ab]±1.155 |
| 8 | PPRC-51 | *Metarhizium anisopliae* | 86.67[ab]±1.764 |
| 9 | DLCO23A | " | 73.00[c]±1.528 |
| vgh10 | DLCO 91 | " | 55.33[d]±2.728 |
| 11 | PPRC-02BC | " | 89.67[ab]±1.202 |
| Tukey's (5%) | | | 2.065 |
| CV (%) | | | 3.1 |

Means within a column followed by the same letter are not significantly different by Tukey's HSD multiple range test at 5% level.

### 3.2. Pathogenicity Test of Entomopathogenic Fungi Against Antestia Bugs

The evaluated isolates showed significant difference against antestia bugs in terms of their pathogenicity (Table 3). The positive control (chemical) recorded complete mortality on the first day of treatments application while, the fungi isolates recorded no mortality on the first day of exposure. On the 3rd days of exposure, positive control registered complete mortality and showed significant difference from the fungi isolates. Isolate PPRC-27J recorded some level of mortality while, others recorded no mortality on the same days after exposure. Six days after application, positive control showed significant difference as compared to tested isolates. On this day all isolates recorded mortality except isolate PPRC-02BC and negative control (Table 3). But, all isolates recorded mortality on 9th days after application and positive control showed significant difference from tested isolates except isolates DELCO-90 and PPRC-27J.

After 12 days of isolates application, PPRC-51, APPRC-0247, PPRC-44BC, DELCO-76, DELCO-90 and PPRC-27J

isolates were not significantly different from positive control. But, they were significantly different from DLCO-56, PPRC-02BC isolates and negative control. Except isolate DLCO-56 and negative control, tested isolates indicated non-significant difference as compared with positive control after 15th days of application (Table 3). After 18 days of application, isolate PPRC-44BC and positive control registered complete mortality and showed significant difference against isolate DLCO-56 and negative control.

Isolates PPRC-44BC and PPRC-27J recorded complete mortality after 21st days of application and similar to positive control which were significantly different from the negative control. From the tested isolates, isolates PPRC-44BC and PPRC-27J recorded maximum mortality followed by DELCO-76 while, the minimum mortality recorded was by isolate DLCO-56. Even though there was difference in pathogenicity levels among tested isolates, the study showed that all the tested entomopathogenic fungi isolates killed antestia bugs and the mortality of antestia bug increased as the exposure time increased (Table 3).

**Table 3.** *Means mortality of antestia bugs treated with Beauveria bassiana and Metarhizium anisopliae isolates over time.*

| Treatments | Means mortality of antestia bugs over exposure time (Days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3rd | 6th | 9th | 12th | 15th | 18th | 21st |
| PPRC-51 | 0 (0.7071)[b] | 0.27 (0.880)[b] | 1.64 (1.462)[b] | 1.93 (1.559)[ab] | 1.93 (1.559)[abc] | 2.79 (1.814)[ab] | 3.23 (1.932)[ab] |
| APPRC-0247 | 0 (0.7071)[b] | 0.27 (0.880)[b] | 1 (1.225)[b] | 1.93 (1.559)[ab] | 2.20 (1.642)[abc] | 2.45 (1.717)[ab] | 3.23 (1.932)[ab] |
| PPRC-44BC | 0 (0.7071)[b] | 0.61 (1.052)[b] | 1.42 (1.386)[b] | 2.95 (1.858)[ab] | 4.32 (2.196)[ab] | 5 (2.345)[a] | 5 (2.345)[a] |
| DLCO-56 | 0 (0.7071)[b] | 0.27 (0.880)[b] | 0.61 (1.052)[b] | 0.87 (1.171)[b] | 0.87 (1.171)[bc] | 0.87 (1.171)[b] | 1.93 (1.559)[ab] |
| DELCO-76 | 0 (0.7071)[b] | 0.27 (0.880)[b] | 0.61 (1.052)[b] | 1.93 (1.559)[ab] | 2.52 (1.739)[abc] | 2.82 (1.822)[ab] | 3.87 (2.091)[ab] |
| PPRC-02BC | 0 (0.7071)[b] | 0 (0.707)[b] | 0.27 (0.880)[b] | 0.83 (1.171)[b] | 1.11 (1.268)[abc] | 1.53 (1.426)[ab] | 2.87 (1.836)[ab] |
| DELCO-90 | 0 (0.7071)[b] | 0.27 (0.880)[b] | 1.93 (1.559)[ab] | 1.93 (1.559)[ab] | 2.20 (1.642)[abc] | 2.45 (1.717)[ab] | 3.47 (1.9932)[ab] |
| PPRC-27J | 0.27 (0.8797)[b] | 0.61 (1.052)[b] | 2.32 (1.678)[ab] | 2.95 (1.858)[ab] | 2.87 (1.836)[abc] | 3.23 (1.932)[ab] | 5 (2.3452)[a] |
| Positive control | 5 (2.3452)[a] | 5 (2.3452)[a] | 5 (2.3452)[a] | 5 (2.3452)[a] | 5 (2.3452)[a] | 5 (2.3452)[a] | 5 (2.3452)[a] |
| Negative control | 0 (0.7071)[b] | 0 (0.707)[b] | 0.61 (1.052)[b] | 0.61 (1.052)[b] | 0.61 (1.052)[c] | 0.61 (1.052)[b] | 1.31 (1.344)[b] |
| Tukey's test | 0.161 | 0.4259 | 0.5108 | 0.5806 | 0.6727 | 0.8295 | 0.5535 |
| CV (%) | 10.6 | 24.4 | 21.9 | 21.8 | 24 | 28.1 | 16.8 |

Means within a column followed by the same letter are not significantly different by Tukey's HSD multiple range test at 5% level. Square root ($\sqrt{x+0.5}$) transformed value in the parenthesis along with mean mortality values.

## 3.3. Median Lethal Time (LT$_{50}$) for the Tested Entomopathogenic Fungi Isolates

The median lethal time (LT$_{50}$), time taken for the death of 50% of adult antestia bug due to the different isolates toxicity was statistical highly significant different. From the tested isolates, isolate PPRC-44BC showed shortest median lethal time (9.98 days) followed by isolate PPRC-27J (10.98 days) (Table 4). On the other hand, the longest median lethal time (19.35 days) was recorded by isolate PPRC-02BC.

**Table 4.** *LT$_{50}$ of tested Entomopathogenic fungal isolates.*

| No. | Isolates | LT$_{50}$ (days) | Lower 95% | Upper 95% | Estimates of parameters (SE) | t pr. |
|---|---|---|---|---|---|---|
| 1 | PPRC-51 | 15.26 | 12.55 | 18.1 | 0.337 | <.001 |
| 2 | APPRC-0247 | 15.79 | 13.12 | 19.0 | 0.339 | <.001 |
| 3 | PPRC-44BC | 9.98 | 7.64 | 11.1 | 0.316 | <.001 |
| 4 | DLCO-56 | - | - | - | - | - |
| 5 | DELCO-76 | 14.49 | 12.34 | 17.8 | 0.336 | <.001 |
| 6 | PPRC-02BC | 19.35 | 17.31 | 26.1 | 0.352 | <.001 |
| 7 | DELCO-90 | 15.06 | 12.28 | 17.7 | 0.336 | <.001 |
| 8 | PPRC-27J | 10.98 | 9.22 | 13.2 | 0.324 | <.001 |

LT$_{50}$ for DLCO-56 isolate was not calculated due to 50% of tested population were not died within twenty one days.

## 3.4. The Effectiveness of EPF Isolates as Influenced by Rates of Application

PPRC-27J and PPRC-44BC isolates evaluated concentrations showed significant difference (Table 5). Tested isolates rate recorded mortality after 6th days of application and positive control showed significant difference from isolates rate on this day after application. But, after nine days of the treatments application, positive control showed non-significant difference except from PPRC-44BC at 1x10⁵ and 1x10⁶ conidia/ml and negative control (Table 5).

Except PPRC-44BC at 1x10⁵ conidia/ml and negative control, tested isolates concentrations non-significantly different as compared with the positive control after 12th days of treatments application. After 15th days of application, PPRC-27J at 1x10⁸ conidia/ml and positive control registered complete mortality and showed significant difference from PPRC-44BC at 1x10⁵ conidia/ml and negative control but not from others treatments. PPRC-27J and PPRC-44BC at 1x10⁸ conidia/ml and positive control registered complete mortality after 18th days of exposure and showed significant difference from negative control and PPRC-44BC at 1x10⁵ conidia/ml (Table 5). Similarly, the maximum rate (1x10⁸ conidia/ ml) for both isolates responded complete mortality after 21st days of application. The result showed that mortality of adult antestia bugs increased as concentration of PPRC-27J and PPRC-44BC isolates increased over exposure time.

**Table 5.** *Means mortality of antestia bugs treated with B. bassiana isolates rate over exposure time.*

| Isolates rate (conidia/ml) | Days of exposure | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3rd | 6th | 9th | 12th | 15th | 18th | 21st |
| PPRC-27J (1*10⁸) | 0 (0.7071)[b] | 1.31 (1.344)[b] | 2.24 (1.655)[ab] | 4.66 (2.271)[c] | 5 (2.345)[c] | 5 (2.345)[b] | 5 (2.345)[b] |
| PPRC-27J (1*10⁷) | 0 (0.7071)[b] | 0.61 (1.052)[b] | 1.93 (1.559)[ab] | 4.66 (2.271)[c] | 4.66 (2.271)[c] | 4.66 (2.271)[b] | 4.66 (2.271)[b] |
| PPRC-27J (1*10⁶) | 0 (0.7071)[b] | 0.27 (0.880)[b] | 1.57 (1.440)[ab] | 3.27 (1.941)[c] | 4.28 (2.187)[c] | 4.28 (2.187)[b] | 4.66 (2.271)[b] |
| PPRC-27J (1*10⁵) | 0 (0.7071)[b] | 1.57 (1.440)[b] | 2.24 (1.655)[ab] | 3 (1.871)[bc] | 3.65 (2.038)[bc] | 3.65 (2.038)[b] | 4 (2.121)[b] |
| PPRC-44BC (1*10⁸) | 0 (0.7071)[b] | 0.27 (0.880)[b] | 2.24 (1.655)[ab] | 3.62 (2.029)[c] | 4.28 (2.187)[c] | 5 (2.345)[b] | 5 (2.345)[b] |
| PPRC-44BC (1*10⁷) | 0 (0.7071)[b] | 0.87 (1.171)[b] | 1.93 (1.559)[ab] | 3 (1.822)[bc] | 3.39 (1.972)[bc] | 4.28 (2.187)[b] | 4.28 (2.187)[b] |

| Isolates rate (conidia/ml) | Days of exposure | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3rd | 6th | 9th | 12th | 15th | 18th | 21st |
| PPRC-44BC ($1*10^6$) | 0 (0.7071)[b] | 0.27 (0.880)[b] | 0.87 (1.171)[a] | 2.79 (1.814)[bc] | 3.10 (1.897)[bc] | 3.87 (2.091)[b] | 4.28 (2.187)[b] |
| PPRC-44BC ($1*10^5$) | 0 (0.7071)[b] | 0.27 (0.880)[b] | 0.27 (0.880)[a] | 0.61 (1.05)[ab] | 1 (1.225)[ab] | 2.24 (1.655)[ab] | 3 (1.871)[b] |
| Positive control | 5 (2.3452)[a] | 5 (2.3452)[a] | 5 (2.3452)[b] | 5 (2.3452)[c] | 5 (2.3452)[c] | 5 (2.3452)[b] | 5 (2.3452)[b] |
| Negative control | 0 (0.7071)[b] | 0 (0.707)[b] | 0 (0.707)[a] | 0 (0.707)[a] | 0 (0.707)[a] | 0.61 (1.052)[a] | 0.61 (1.052)[a] |
| Tukey's test | 0.161 | 0.458 | 0.655 | 0.505 | 0.5271 | 0.42 | 0.28 |
| CV | 10.6 | 23.2 | 26.5 | 16.4 | 16.1 | 12 | 7.9 |

Means within a column followed by the same letter are not significantly different by Tukey's HSD multiple range test at 5% level. Square root ($\sqrt{x+0.5}$) transformed value in the parenthesis along with mean mortality values.

### 3.5. Median Lethal Time (LT$_{50}$) of Effective EPF Isolates at Different Concentrations

PPRC-27J and PPRC-44BC isolates rate showed statistically significant difference median lethal time (LT$_{50}$) (Table 6). The shortest median lethal time (LT$_{50}$) was 9.28 days by PPRC-27J at $1x10^8$ conidia/ml followed by 9.47 days of PPRC-27J at $1x10^7$ conidia/ml while, LT$_{50}$ of PPRC-44BC at $1x10^8$ conidia/ml was 9.61 days. On the contrary, the longest median lethal time (LT$_{50}$) was 19.23 days by PPRC-44BC at $1x10^5$ conidia/ ml.

**Table 6.** *Median lethal time (LT$_{50}$) of Beauveria bassiana isolates at different concentrations.*

| Isolates rates (conidia/ml) | LT$_{50}$ | Lower 95% | Upper 95% | Estimates of parameters (SE) | t pr. |
|---|---|---|---|---|---|
| PPRC-27J ($1x10^8$) | 9.28 | 8.55 | 10.08 | 0.309 | <.001 |
| PPRC-27J ($1x10^7$) | 9.47 | 8.60 | 10.17 | 0.315 | <.001 |
| PPRC-27J ($1x10^6$) | 10.15 | 8.63 | 11.93 | 0.330 | <.001 |
| PPRC-27J ($1x10^5$) | 10.19 | 8.67 | 11.88 | 0.329 | <.001 |
| PPRC-44BC ($1x10^8$) | 9.61 | 8.16 | 11.27 | 0.327 | <.001 |
| PPRC-44BC ($1x10^7$) | 11.76 | 10.05 | 13.74 | 0.336 | <.001 |
| PPRC-44BC ($1x10^6$) | 12.29 | 10.51 | 14.36 | 0.339 | <.001 |
| PPRC-44BC ($1x10^5$) | 19.23 | 17.53 | 20.54 | 0.431 | <.001 |

### 3.6. Median Lethal Concentration (LC$_{50}$) of Effective EPF Isolates

The median lethal concentrations (LC$_{50}$ and LC$_{90}$) of PPRC-27J and PPRC-44BC isolates rate showed statistically significant difference (Table 7). The lower median lethal concentrations (LC$_{50}$ and LC$_{90}$) were $8.87x10^3$ and $6.98x10^5$ conidia/ml, respectively by isolate PPRC-27J. While, the higher median lethal concentrations (LC$_{50}$ and LC$_{90}$) were $7.19x10^4$ and $2.12x10^6$ conidia/ml, respectively by isolate PPRC-44BC.

**Table 7.** *Median lethal concentrations (LC$_{50}$ and LC$_{90}$) of effective B. bassiana isolates tested against Antestia bugs.*

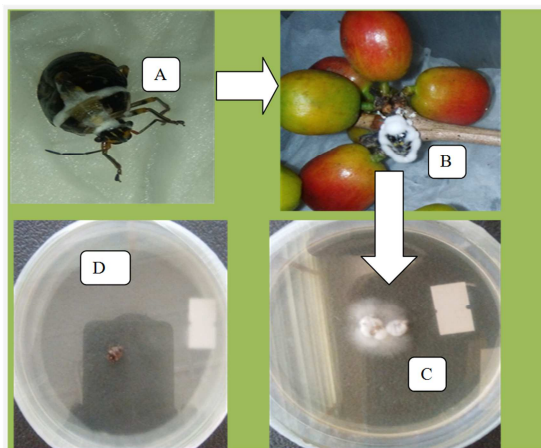| Isolates | LC$_{50}$ (conidia/ml) | LC$_{90}$ (conidia/ml) | Slope | P-value |
|---|---|---|---|---|
| PPRC-27J | $8.87x10^3$ | $6.98x10^5$ | 0.675 | 0.25031 |
| PPRC-44BC | $7.19x10^4$ | $2.12x10^6$ | 0.852 | 0.669158 |



**Figure 3.** *Antestia bugs treated and untreated with EPF isolates. A) Mycosed antestia bug, B) overgrowth of EPF on antestia bug, C) Re-isolation of EPF from mycosed antestia bug and D) untreated antestia bug with EPF (negative control).*

## 4. Discussion

The study showed that PPRC-44BC and PPRC-27J isolates showed complete mortality against antestia bug. It indicates PPRC-44BC and PPRC-27J isolates were more pathogenic than others isolates. This may be related with genetic variability of the isolates with regard to virulence against the pest and susceptibility of the host. The study agreed with earlier authors who suggested that it may be a result of the genetic variability of the isolates with regard to virulence [36, 37]. Alves also reported that the intrinsic qualities of the pathogen, the susceptibility and/ or the natural resistance of host insect itself, is another factor in the pathogenesis of an isolate [37]. This behavior is related to virulence and the production of secondary metabolites that influence the ability of the pathogen to cause disease [38, 39].

Findings related to antestia bug and EPF is scarce. However, studies on EPF and other pests of economic importance can be mentioned. For example Sanjaya *et al.*

reported that the variation in virulence among different fungal isolates to *Tetranychus kanzawai* may be associated with the enzymes produced by each isolates [40, 41]. The physiological state of the host insect also plays a role in pathogenicity in the treated population [42]. Sanjaya *et al*. also indicated that the pathogenicity of a fungal isolate is dependent on its ability to attach on and penetrate the host cuticle as well as to replicate within the host, usually in the hemocoel [40]. Robert and Leger reported that secondary metabolites production for instance, toxins such as destruxins and beauvericin, present in *M. anisopliae* and *B. bassiana,* respectively may contribute to the observed variation in virulence [43]. The virulence of an EPF is also associated with their ability to produce toxic substances, which interfere not only with the normal host development and metamorphosis but in some cases with the immune system until the host ultimately dies from fungal infection [40].

The study indicated that *B. bassiana* species, PPRC-44BC and PPRC-27J isolates were more pathogenic than *M. anisopliae* species. This finding agrees with Nahayo and Bayisenge, and Sevim *et al*. who conducted study on *B. bassiana* fungus to control antestia bugs and concluded the pathogenecity of the fungus against antestia bugs [32, 44]. Erler and Ates, and Tamiru *et al*. also indicated that *B. bassiana* product was more effective than the formulations of *M. anisopliae* product [45, 44]. Similarly, Samuels *et al*. reported that *B. bassiana* as one of the most promising biological control agents for coffee berry borer and an effective component within an integrated pest management program in Mexico [46]. Sevim *et al*. recommended *B. bassiana* KTU-24 as biocontrol agent against a number of forest pests based on its high virulence in Turkey [47]. Feng *et al*. also indicated that *B. bassiana* has proved to be competitive with chemical insecticides for forest and farm insect pests in China [48]. And Tamiru *et al*. promoted PPRC-56 isolate, *B. bassiana* against antestia [44].

As Wraigh and Ramos reported, a good control means statistically significant reductions in pest numbers or damage of 75% or more, compared to an untreated control [49]. Further, they stated that a fair control includes those with significant reductions of 50 - 74%, and any non-significant reductions of over 50% and the poor control group includes any results with less than 50% reduction. But, this study showed that PPRC-44BC and PPRC-27J isolates registered complete mortality against Antestia bugs. This revealed PPRC-44BC and PPRC-27J isolates can be considered as good control options of antestia bugs and needs further investigations.

The study indicated that PPRC-44BC and PPRC-27J isolates showed promising results causing lower median lethal time ($LT_{50}$). The lifecycle study indicated that the average longevity, for the female and male bugs was 187±7.8 and 135±10 days, respectively [27]. PPRC-44BC and PPRC-27J isolates showed complete mortality after 18th and 21st days of isolates application, respectively. But, Tamiru *et al*. reported that PPRC-56 and PPRC-2 killed antestia bug above 70% within twenty days [44]. Kooyman *et al*. reported that 80% reduction in grasshopper populations was recorded in treated plots after twenty-one days of isolates application [50]. Lomer *et al*. indicated that entomopathogenic fungi biopesticide kills 70 - 90% of treated locusts within 14-20 days [51]. The mortality of the brown marmorated stink bug after 12 - 28 days ranged from 67 to 100% and 40 to 88% by *Beauveria* and *Metarhizium* isolates, respectively [52, 47, 53].

Different study reported that EPF isolates can serve pest control for long period of time without affecting the natural enemies while; the chemical control is for short period of time for as soon as the pest can develop resistance against it [51]. Mensah & Young, and Bayu & Prayogo also reported that use of *B. bassiana* is safe to the predators and effectively increased the yield as compared with the application of chemical insecticide on mungbean insect pest and *Bemisia tabaci* on cotton crop, respectively [54, 55]. This indicates that PPRC-44BC and PPRC-27J isolates are good candidates for further study against antestia bug.

This study revealed that all fungal isolates used infected and caused mortality at all tested concentrations. The mortality of adult antestia bugs increased as concentration of PPRC-44BC and PPRC-27J isolates increased over exposure time. It may be higher concentrations of the isolates have a chance to attach to antestia bug easily and kill it. On other hand, since antestia bug is big in size it required higher concentration for kill. This is in-line with Tamiru *et al*. who reported PPRC-56 and PPRC-2 recorded higher mortality of antestia bug at higher concentration [44]. The study agreed with Sanjaya *et al*. who found the maximum mortality of the tested insect species at higher concentration ($1 \times 10^8$ conidia/ml) [40, 47, 56]. Muller documented the infection and killing speed of *Locuslana pardalina* (Walker) observed the highest mortality at highest concentration while mortality decreased as concentrations decrease [57].

Similarly, Bugti *et al*. reported that dose dependency in fungal disease normally indicates a higher density of conidia and often results in a faster control of targeted insects [56]. This study is also in-line with Younas *et al*, who showed *Beauveria spp*. isolates at higher concentration registered higher mortality [40, 58]. Addisu *et al*. also suggested that the use of EPF at higher concentrations is an eco-friendly effective mycoinsecticides that causes more than 95% mortality of *Macrotermes* [59]. All the fungal products varied in ability to infect June beetle larvae, and their impact on mortality largely depended on the conidial concentrations applied and the elapsed time after application [45]. On other hand, Bugti *et al*. reported that to control large insect pest populations higher conidial concentrations are more effective than lower concentrations to avoid pest populations reaching economic injury levels [56]. Mouatcho also indicated that mortality rates are associated with length of exposure to conidia because increasing length of exposure increases the potential for acquiring a lethal fungal infection [42].

The result showed that the median lethal time ($LT_{50}$) decreased as isolates concentration increased over exposure time. PPRC-27J and PPRC-44BC isolates showed promising result while, EPF infection usually takes 5 to 10 days to kill

an insect [60]. Addisu *et al.* reported that the isolate had $LT_{50}$ ranging from 7.74 days in *M. anisopliae* isolate PPRC-2 to 8.80 days in *B. bassiana* isolate 9609 [59]. Cheraghi *et al.* revealed that *B. bassiana* and *M. anisopliae* are highly pathogenic to *An. funestus* adult females, with 99% mortality achieved within 14 days post infection [42, 61]. But compared to synthetic chemical insecticides, major drawback on the development of EPF as a biological control agent is its slower killing action [62]. PPRC-27J and PPRC-44BC isolates showed promising result for further investigations as biocontrol agent. In conformity, Sevim *et al.* reported that the $LC_{50}$ values of isolate *B. bassiana* KTU-24 were $5.51 \times 10^5$ and $3.96 \times 10^5$ conidia ml$^{-1}$ against adults and nymphs, of *C. ciliate* and considered as promising candidate for further investigations as a biocontrol agent [47].

## 5. Conclusions

The study was conducted to evaluate the effectiveness of EPF isolates under laboratory condition against antestia bug. Eleven EPF isolates (DLCO 12, APPRC 0247, DLCO 90, DLCO 56, DLCO 76, PPRC-44BC, PPRC-27J, PPRC-51, DLCO23A, DLCO 91 and PPRC-02BC) were evaluated for germination test and the better germinated eight isolates (APPRC 0247, DLCO 90, DLCO 56, DLCO 76, PPRC-44BC, PPRC-27J, PPRC-51 and PPRC-02BC) were tested for pathogenicity against antestia bug. Additionally the more effective, PPRC-27J and PPRC-44BC isolates of *B. bassiana* species were evaluated for concentrations efficacy. And the experiment was laid out by CRD with three replications.

The tested fungal isolates showed increased mortality of antestia bug as exposure time increased which means the higher mortality were recorded at end of the exposure time. From evaluated fungal isolates, PPRC-27J and PPRC-44BC registered complete mortality against antestia bug and shorter median lethal time ($LT_{50}$) (9.98 and 10.98 days, respectively). Also the mortality of antestia bugs increased as concentration and exposure time of the isolates increased. The higher concentration ($1 \times 10^8$ condia per ml) showed complete mortality of the pest within short period of time relative to the lower concentrations. PPRC-27J isolate registered lower median lethal concentrations ($8.87 \times 10^3$ and $6.98 \times 10^5$ conidia/ml) for $LC_{50}$ and $LC_{90}$, respectively. But PPRC-44BC showed higher median lethal concentrations ($7.19 \times 10^4$ and $2.12 \times 10^6$ conidia/ml) for $LC_{50}$ and $LC_{90}$, respectively. These isolates showed similar result with the positive control but their difference was the time taken to achieve complete mortality of the pest.

PPRC-27J and PPRC-44BC isolates showed promising result under laboratory condition. But their efficacy under field conditions, on antestia bug natural enemies, different developmental stages of the pest and application methods needs further investigations. Furthermore, the formulation of effective EPF isolates against antestia bug requires research. The isolation of EPF, others microbial, entomopathogenic bacteria and entomopathogenic nematode could also be considered for further investigations.

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