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SUSCEPTIBILITY OF *SITOPHILUS ZEAMAIS* (MOSTCH.) (COLEOPTERA: CURCULIONIDAE) TO *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE*

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ABSTRACT: Evaluation of eleven *Metarhizium anisopliae* and six *Beauveria bassiana* isolates against the maize weevil *Sitophilus zeamais* was conducted under laboratory with the objectives of identifying the most virulent locally available fungal isolates, and determining the dose mortality response. The pathogenicity (virulence) of the entomopathogenic fungi was determined using LT_{50} and percent mortality at the conidial concentration of 1×10^8 ml^{-1}. The most virulent isolates of *M. anisopliae* (PPRC-2, PPRC-14 and PPRC-51) and *B. bassiana* (PPRC-GG and PPRC-HH) caused 84.4% to 98.3% mortality to *S. zeamais*. These isolates had LT_{50} ranging from 3.8 to 4.3 days. The dose response study with the isolates PPRC-2, PPRC-14 and PPRC-51 at doses ranging from 1×10^4 to 1×10^8 conidia ml^{-1} revealed susceptibility of the *S. zeamais* with mortality ranging from 25 to 95.5%. There was an increase in the resulting LT_{50} from 4.0 to 11.9 days with a decrease in conidial concentration from 1×10^8 to 1×10^4 conidia ml^{-1}. PPRC-2 showed the lowest LC_{50} (1.46x 10 conidia ml^{-1}) followed by PPRC-14 and PPRC-51 (3.5×10^5 and 3.9×10^5 conidia ml^{-1}, respectively). In conclusion, the isolates PPRC-2, PPRC-14, PPRC-51, PPRC-GG and PPRC-HH, can be considered as a potential candidates in the development of mycoinsectisdes against *S. zeamais*.

Key words/phrases: ***Beauveria bassiana*, *Metarhizium anisopliae*, mortality, LC_{50}, LT_{50}**

INTRODUCTION

The maize weevil, *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae), is one of the most serious cosmopolitan pest of stored cereal grain, especially of maize (*Zea mays* L.), in tropical and sub-tropical regions (Throne, 1994). Grain yield losses up to 40% were reported on stored maize due to *S. zeamais* in Africa (Meikle *et al*., 2001). In Africa, where subsistence grain production supports the population, such grain losses may be substantial. In addition to grain weight loss, pests of stored grain also cause secondary fungal infection, resulting in a reduction in seed vigor, quality and commercial value (Meikle *et al*., 2001).

Synthetic chemical insecticides have been widely used for the control of pests of stored grain, particularly *S. zeamais*. The widespread use of insecticides for the control of stored-product insect pests is of global concern with respect to environmental hazards, insecticide resistance development, chemical residues in foodstuffs, side effects on non-target organisms and the associated high costs (Cherry *et al*., 2005). To this effect, the increased public awareness and concern for environmental safety has directed research to the development of alternative control strategies such as the use of entomopathogenic fungi against stored-product insect pests.

Variations in pathogenicity (virulence) among isolates against stored product insects have been previously reported in assays with *B. bassiana* and *M. anisopliae* on *S. zeamais* (Adane Kassa *et al*., 1996; Hildago *et al*., 1998; Adane Kassa, 2003), *Sitophilus oryzae* (Dal Bello *et al*., 2001; Sheeba *et al*., 2001; Padin *et al*., 2002), *Prostophanus truncatus* (Miekel *et al*., 2001; Adane Kassa, 2003), *Sitophilus granarius* (Athanassiou and Steenberg, 2006), *Rhizopertha domonica* (Lord, 2001), *Caryedon serratus* (Ekesi, 2001), *Cryptolestes ferrugeneus* (Lord, 2001), *Oryzophilus surrinamensis* (Thorne and Lord, 2004), *Plodia interpunclella* and *Ephestia kuehniella* (Bischoff and Reichmuth, 1997).

One of the first steps in development of an entomopathogenic fungus for the bio-control is selection of virulent strain (Tadele Tefera and Pringle, 2007). Little effort has been made to evaluate the potential of entomopathogeic fungai for biological control of *S. zeamais*. The objectives of the present study, therefore, were to identify the

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most virulent locally available fungal isolates of *B. bassiana* and *M. anisopliae* against the maize weevil *S. zeamais* and to determine the concentration-mortality response of the most virulent isolates.

MATERIALS AND METHODS

Rearing S. zeamais

About 1500 unsexed adult maize weevils collected from the Bako Agricultural Research Centre (BARC), Ethiopia, maize store were placed in 10 four-litre plastic containers each containing 5 kg of maize. The top of each plastic container was covered with nylon mesh fastened tightly with elastic bands. The insects were allowed for two-week oviposition periods before all adults were removed. After two weeks of oviposition, all *S. zeamais* were removed and transferred to fresh maize grain in another plastic container. Such removal and transfer of *S. zeamais* to fresh maize grain were repeated for three weeks in order to produce sufficient number of progenies *S. zeamais* of the same batch for the subsequent experiments. Each plastic container where *S. zeamais* oviposited was kept for progeny emergence. Thirty-five days after introduction of weevils in each plastic container, emerging progenies of *S. zeamais* were removed daily until the progenies emergence ceased; and those emerged on the same day were transferred to fresh grain in plastic containers with lids and were kept at the experimental conditions (22 ± 1°C) until sufficient number of *S. zeamais* were obtained.

Maize grain

Insecticide untreated and clean, infestation and dockage free maize grain of variety BH-540 harvested in off-season of 2007 was obtained from the BARC. The initial grain moisture content and seed viability were 12.5% and 100%, respectively. To avoid risk of secondary infestation grains were stored in deep freeze at -20°C for 21 days before use. It was then kept for two weeks at the experimental conditions for acclimatization of moisture and temperature (Abraham Tadesse, 2003).

Fungal isolates

A total of seventeen fungal isolates comprising eleven *M. anisopliae* and six *B. bassiana* were obtained from the Ethiopian Institute of Agricultural Research (EIAR), Plant Protection Research Centre (PPRC), Ambo. The isolates had been isolated from different arthropods in different agro-ecological zones of Ethiopia. However, one isolates, ICIPE-30, was obtained from the International Centre of Insect Physiology and Ecology, Kenya. Details of these isolates are given in Table 1. The initial cultures of all isolates were stored at 4°C and sub-culturing was made for the present work.

Table 1. List of *Metarhizium anisopliae* and *Beauveria bassiana* isolates tested.

Fungal species	Isolate code	Host arthropod	Specific site of origin	Altitude
M. anisopliae	PPRC-2	*Pachnoda interrupta*	Ashan	2400
M. anisopliae	PPRC-4	*P. interrupta*	Shoa Robit	1875
M. anisopliae	PPRC-14	*P. interrupta*	Dedeaa	2400
M. anisopliae	PPRC-19	*P. interrupta*	Rufe Kure	1750
M. anisopliae	PPRC-27	*P. interrupta*	Dedeaa	2400
M. anisopliae	PPRC-29	*P. interrupta*	Gobenayetu	2400
M. anisopliae	PPRC-51	*P. interrupta*	Shoa Robit	2400
M. anisopliae	PPRC-56	*P. interrupta*	Berber	1925
M. anisopliae	PPRC-61	Spider (Arachnida)	Awaketu	1750
M. anisopliae	PPRC-66	Spider (Arachnida)	Awaketu	1950
M. anisopliae	ICIPE-30	Tse Tse fly	Kenya	Unknown
B. bassiana	PPRC-9501	Chafer grub	Tikur Inchini	1950
B. bassiana	PPRC-9609	*Blosynus mugulosus*	Dila	2030
B. bassiana	PPRC-9614	Ground beetle	Awassa	1500
B. sp.	PPRC-9615	Spider (Arachnida)	Awassa	2450
B. bassiana	PPRC-GG	Coleoptera (adult)	Ashange	2400
B. bassiana	PPRC-HH	Coleoptera (adult)	Ashange	2400

To produce inocula for experiments, slant cultures of the seventeen isolates from the collections were sub-cultured onto Sabouraud dextrose agar with yeast extract (SDAY) in to a Petri dish sealed with Para film. Cultures were incubated at 27°C and 75% RH for 20-days. The surface of 20-day old cultures was scrapped with a sterile scalpel and suspended in aqueous solution of 0.01% Tween 80. The fungal suspension was vortexed for one minute to break up the conidial chains or clumps and filtered through several layers of sterile cheesecloth to remove mycelia. The dose of conidia in the filtrate was estimated using heamocytometer under a light microscope (40x magnifications). A standard conidial suspension of $1x10^8$ conidia ml^{-1} was prepared for each isolate.

Viability of conidia was estimated using a dilution plate count method. For each isolate, one ml of suspension containing $1x10^4$ conidia ml^{-1} were spread plated over SDAY plates with sterile glass rod and sealed with Para film. Four Petri dishes of each fungal isolate were then incubated at 27°C and 75% RH for 24 hrs. Germination rate was determined microscopically for at least 100 conidia from random field views for each isolate. Conidia were considered to have germinated when the germ tube was at least as long as the width of the germ tube. The germination of conidia of the isolates exceeded 80%.

Bioassays

Single concentration assay

In this assay seventeen isolates were assayed for pathogenicity against the maize weevil. The weevils were collected from rearing jars, placed in a Petri dish and mixed thoroughly to facilitate random selection of the insects. Laboratory reared 7 to 10 days old adult weevils as described above were used. Fifteen weevils were then introduced to each Petri dish with filter paper inside. For each isolate an aqueous suspension containing 1.0×10^8 conidia ml^{-1} were prepared in 0.01% Tween 80. Inoculation of each batch of weevils was made by directly spraying one ml of 1.0×10^8conidia ml^{-1} of each isolate using a hand held spray atomizer. This conidial concentration was chosen based on our previous report (Tadele Tefera and Prinlgle, 2007). For the control group, the same numbers of maize weevils were treated with sterile distilled water with 0.01% Tween 80.

The treated insects and the control were kept in Petri dishes with perforated lid for 24 h without food at 27°C and 70 ± 5% RH. This was done to avoid dislodging of conidia by the maize kernels. After 24 h, insects were transferred to glass jar (250 ml capacity) containing 200 g of cracked and disinfected maize kernels and the jars were covered with a perforated lid. The treatments were maintained at 27°C and 70 ± 5% RH for 10 days. All the treatments and the controls were replicated four times in a completely randomized design.

Mortality was recorded daily for ten days. An observation of each jar was done by emptying the contents of each jar onto white paper to identify dead individuals. The dead adults collected were immediately submerged in to 95% ethanol for one minute and washed in sterile distilled water for five minutes to remove saprophytes and all conidia found on the outer surface of the insect body. The disinfected cadavers were then allowed to dry for ten minutes. This step was added to ensure that mycosis observed on the surface of the cadavers would not be attributed to spores used during the treatment but rather to growth from the interior to the exterior of the insect after colonization of internal organs. Cadavers were then held under high humidity on Petri dishes containing damp filter paper to provide sufficient humid conditions to promote fungal outgrowth. Petri dishes were sealed with Para film to maintain greater than 95% RH and were incubated in the dark at 27°C. Insects were considered mycosed when growth of the fungus was visible on the external surface of the insect's cuticle and those which showed hyphal growth characteristic of the entomopathogenic fungi were recorded as infected.

Multiple concentration assays

The dose responses of the isolates PPRC-2, PPRC-51 and PPRC-14 were determined. The isolates were selected based on the results of single dose assay in which they caused at least 95% mortality (Table 2). Serial dilutions were made to obtain five different conidial doses ($1x\ 10^4$, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1}) with 0.01% Tween 80. The conidial suspensions were applied to weevils following the same procedure as described above. Fifteen newly emerged adult maize weevils were used for each dose of each isolate. The control group was treated with sterile distilled water containing 0.01% Tween 80. Petri dishes containing treated and control insects were sealed with Para film and incubated at 27°C. The treated insects and the controls were provided with untreated cracked maize grains 24 hours after treatment application. All treatments and the controls were replicated four times and arranged in a completely randomized design. Insect mortality was recorded daily for ten days. Dead insects were removed and placed in a Petri dish with moist filter paper. Fungal infection was confirmed after observing sporulated cadavers under stereomicroscope.

Table 2.. Mean LT_{50} of *S. zeamais* 10 days after treatment with the isolates of *M. anisopliae* and *B. bassiana* at the rate of $1x10^8$ conidia ml^{-1}.

Fungal species	Isolate code	LT_{50} (days)	95% CI	Slope ±S.E	Chi-square	P-value
M. anisopliae	PPRC-2	3.9±0.723	1.5–5.33	0.77±0.055	7968.5	<0.001
M. anisopliae	PPRC-4	10.2±0.361	8.3–15.03	0.20±0.2	31.54	<0.001
M. anisopliae	PPRC-14	4.2±0.61	3.3–5.02	0.65±0.44	494.48	<0.001
M. anisopliae	PPRC-19	9.6±0.353	8.6–11.43	0.22±0.024	23.3	0.003
M. anisopliae	PPRC-27	23.6±0.347	16–52.6	0.17±0.03	7.47	0.49
M. anisopliae	PPRC-29	15.6±0.401	12.4–23.6	0.21±0.29	8.13	0.42
M. anisopliae	PPRC-51	4.3±0.522	3.5–45.1	0.57±0.04	189.3	<0.001
M. anisopliae	PPRC-56	6.6±0.44	6.3–7.03	0.36±0.027	27.2	0.001
M. anisopliae	PPRC-61	16.5±0.64	12.9–27.6	0.22±0.04	12.6	0.122
M. anisopliae	PPRC-66	10.2±0.347	8.3–14.9	0.21±0.02	29.2	<0.001
M. anisopliae	ICIPE-30	7.8±0.398	6.9–9.26	0.28±0.025	34.9	<0.001
B. bassiana	PPRC-9501	7.9±0.345	6.5–9.373	0.25±0.02	38.63	<0.001
B. bassiana	PPRC-9609	17.8±0.668	13.6–32.55	0.22±0.34	2.62	0.956
B. bassiana	PPRC-9614	30.9±0.504	18.5–139.6	0.17±0.038	2.2	0.974
B. bassiana	PPRC-9615	22.5±0.368	15.6–48.13	0.16±0.027	7.16	0.519
B. bassiana	PPRC-GG	4.0±0.282	3.8–5.56	0.33±0.24	73	<0.001
B. bassiana	PPRC-HH	4.2±0.44	3.3–5.03	0.50±0.32	154.5	<0.001

CI = Confidence interval; SE = Standard error.

Statistical analysis

Mortality data were corrected for the control mortality $\%CM = \frac{(\%T - \%C)}{(100 - \%C)} * 100$; where CM is corrected mortality, T is mortality in treated insects and C is mortality in untreated insects (Abbott, 1925). The data were arcsine transformed to stabilize variances. Mortality was analyzed using one-way analysis of variance (ANOVA) ($P < 0.001$) with PROC ANOVA (SAS Institute, 1999). Least significance difference (LSD) test was used to separate means. The LT_{50} (Lethal time required to kill 50% of the treated insect population) was determined using probit analysis.

Mortality was corrected for the control (Abbott, 1925). LC_{50} (Lethal dose of conidia required to kill 50% of the treated insect population) was determined using probit analysis. An arcsine transformation of percentage mortality was performed. Mortality data were analyzed using General Linear Model's Procedure (PROC GLM). Least significance difference (LSD) test was used to separate means (SAS Institute, 1999).

RESULTS

Single concentration assay

No mortality observed in control insects. There were significant differences ($F = 13.923$, $df = 17$, $P < 0.001$) between fungal treatments in causing mortality to *S. zeamais* (Fig. 1). The isolates PPRC-9614, PPRC-9609, PPRC-27, PPRC-29, PPRC-61, and PPRC-9615, were the least virulent causing mortality, 13.3% to 31.7%, whilst the isolates PPRC-4, PPRC-19, PPRC-56, ICIPE-30 and PPRC-66, were moderate in virulence inducing 40% to 73% mortality. However, PPRC-2, PPRC-51, PPRC-14, PPRC-GG and PPRC-HH were the most virulent isolates with 84.8% to 98.32% mortality (Fig. 1).

Though all isolates induced mortality, the single dose time–mortality assay demonstrated important differences in LT_{50} values between the isolates (Table 2). The mortality rate of the three groups of the isolates was highly related to LT_{50}. The isolates which had the least LT_{50} values, had the highest mortality rates and vice versa. The LT_{50} ranged from 3.8 to 30.9 days. The most virulent, moderately and weakly virulent isolates had LT_{50} ranging from 3.8 to 4.3, 6.6 to 10.2 and 16.5 to 30.9 days, respectively. The slopes of the lines are different ($P < 0.05$) from zero except for the isolates PPRC-27, PPRC-29, PPRC-61, PPRC-9609, PPRC-9614 and PPRC-9615.

Multiple concentration assays

The LC_{50} of PPRC-2 (1.4×10^5 conidia ml^{-1}) was lower than that of PPRC-51 (3.9×10^5 conidia ml^{-1}) and PPRC-14 (3.5×10^5 conidia ml^{-1}) (Table 3). However, there was an overlap in fiducial limits

between the isolates. Therefore, the differences in the LC_{50} values were not significant.

There were also significant differences (F = 163.6, df = 2, P<0.01) between isolates in mortality (F = 6.04, df = 4 P<0.01) (Table 4). No mortality was observed in control insects. The cumulative mortality of all isolates at different rates indicated the dose dependent mortality of *S. zeamais*. The *S. zeamais* mortality at the highest (1×10^8 conidia ml^{-1}) and lowest (1×10^4 conidia ml^{-1}) conidial concentration ranged from 93% to 95% and 25% to 28.33%, respectively (Table 4). At the three higher concentrations (dosages), PPRC-2 caused significantly higher mortality than the other isolates. At low application concentration (1×10^4 and 1×10^5 conidia ml^{-1}), the mortality level was significantly decreased. There was an increase in LT_{50} with a corresponding decrease in conidial concentration. The LT_{50} value ranged from 4.2 to 4.6 days and 11.7 to 11.8 days for the highest and the lowest conidia concentration, respectively.

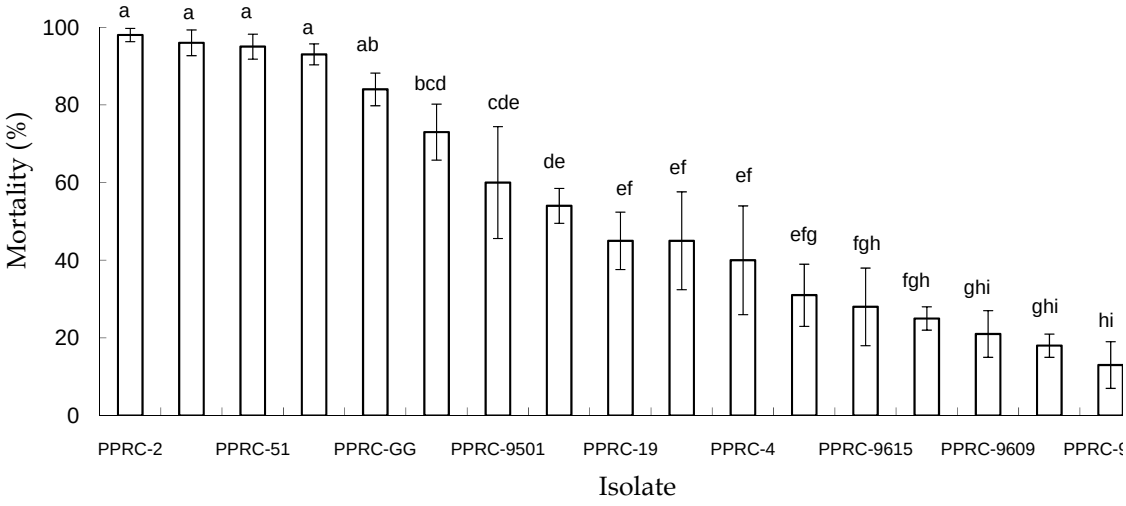

Fig. 1. Mean percentage mortality (±SE) of *S. zeamais* 10 days after treatment with the isolates of *M. anisopliae* and *B. bassiana* at the rate of 1×10^8 conidia ml^{-1}.

Table 3. LC_{50} of three isolates of *M. anisopliae* against *S. zeamais*.

Fungal isolates	LC_{50}	95% Fiducial limit	Slope (SE)	X^2 (df = 3)
PPRC-2	1.46×10^5	$6.4\times10^4 - 2.8\times10^5$	0.064	4.33
PPRC-14	3.5×10^5	$1.7\times10^5 - 8.3\times10^5$	0.063	3.35
PPRC-51	3.9×10^5	$1.7\times10^5 - 6.8\times10^5$	0.059	4.19

Table 4. Mortality and LT_{50} of *S. zeamais* six days after treatment with different conidial concentration of *M. anisopliae*.

Conidial Conc. ml^{-1}	Mortality (±SE)*			LT_{50} (days)		
	PPRC-2	PPRC-14	PPRC-51	PPRC-2	PPRC-14	PPRC-51
1×10^4	28.3 ± 4.9^e	25.0 ± 3.19^e	26.6 ± 4.7^e	11.7	11.9	11.8
1×10^5	38.3 ± 3.1^d	30.0 ± 1.92^d	38.3 ± 3.19^d	9.6	10.9	9.6
1×10^6	76.6 ± 4.3^c	61.6 ± 6.3^c	51.6 ± 1.67^c	5.9	6.9	7.9
1×10^7	81.6 ± 3.19^b	76.6 ± 4.3^b	70.0 ± 4.3^b	5.5	5.9	5.5
1×10^8	95.0 ± 3.19^a	93.3 ± 2.7^a	93.3 ± 2.7^a	4.2	4.8	4.6

*Mean values followed by the same letter within a row are not significantly different (P<0.05) using the least significance difference (LSD) test.

DISCUSSION

The presented study demonstrated variation in pathogenicity among the fungal isolates. The cause for such variation in pathogenicity possibly attributed to production of toxic substances by the isolates, immune system of the host and the bioassay temperature. Adane Kassa *et al.* (1996) and Tadele Tefera (2004) reported that the pathogenicity of some of these isolates was affected when the temperature was below 25°C. Development of fungal pathogen within hosts can be influenced not only by immune reaction of the host but also indirectly by the hosts' diet (Tadele Tefera and Pringle, 2003; Moorehouse *et al.,* 1993). The difference in the ability of an isolate to overcome the host defence system may be reflected in the increased insect survival following treatment as well as the higher $LT_{50.}$ The differences observed in LT_{50} values in isolates tested here could reflect genetic and physiological differences between isolates or factors such as toxins or characteristics of the insect host. Moorehouse *et al.* (1993) demonstrated that LT_{50} was correlated with spore dose and it is, therefore, only possible to compare directly the result of different bioassay systems if the dose received by individual insects is known.

Moino *et al.* (1998) and Dal Bello *et al*. (2006) reported greater mortality of stored grain pests inoculated with Beauveria isolates than Metarhizium isolates. In contrast to these findings, the present study clearly demonstrated that when either *B. bassiana* or *M. anisopliae* were applied to *S. zeamais*, the later caused higher mortality and shorter median survival time. For instance, *M. anisopliae* isolates (PPRC-2, PPRC-14 and PPRC-51) caused higher mortality than *B. bassiana* isolates (PPRC-HH and PPRC-GG). The differences are might be attributed to the virulence of the isolated tested.

The study indicated that the isolates were not specific to their original host as none of the isolates tested were from *S. zeamais*. Todrova *et al.* (2002) also indicated that the original host was not found to be a reliable indicator of the pathogenicity of a specific fungus to a specific host. Tadele Tefera (2004) also reported high pathogenicity of the isolates PPRC-4 and PPRC-HH to the spotted stem borer *Chilo partellus*. However, Talaei-Hassanloui *et al.* (2006) stated that the most virulent isolates to a host are those that are isolated from the same or related host species. It is agreed that *B. bassiana* and *M. anisopliae* have wide host ranges and their pathogenicity also vary according to their host. There is increasing evidence that habitat selection drives the pathogenicity of *B. bassiana* and *M. anisopliae* (Bidochka *et al.,* 2000). Thus, results from this study indicate that screening of potential isolates should not be limited to those isolated from the original host.

The isolate PPRC-2 was consistently more virulent than PPRC-14 and PPRC-51. Thus, considering both the LT_{50} and LC_{50} values, these isolates may be considered among the most virulent isolates tested against adult maize weevils in this study. The observed high mortality at 1×10^{8} and 1×10^{7} conidia ml^{-1} seem to be attributable to the increased number of conidia that adhered to an insect at these doses. Mortality occurred at all doses for all isolates but to a limited (25% to 28% mortality) extent at low doses. This low mortality appeared to stem from the slow development of infection at lower dose (Yoon *et al.,* 1999). Large doses containing the host causes death in few days, whereas, low doses may take two or more weeks to kill (Hidalgo *et al.,* 1998). Adane Kassa *et al.* (1996) evaluated the pathogenicity of ten *B. bassiana* isolates to the maize weevil and found that one of the isolates caused 88% mortality within eight days at $1x10^{4}$ conidia ml^{-1} indicating very virulent isolates could cause high mortality at lower doses. High dose requirement for stored grain pests has been reported for *T. castaneum* (Padin *et al.,* 2002) and Cheryy *et al.* (2005) on *Callosobruchus maculatus*. However, even at lower doses, *B. bassiana* has shown to have a comparatively better performance on *R. dominica*, *O. surinamensis* and *Cryptolestes ferrugineus* (Lord, 2001). Adane Kassa (2003) also reported that *B. bassiana* isolates appeared virulent to *S. zeamais* only at doses higher than $1x10^{7}$ conidia ml^{-1} and variability within the different *B. bassiana* isolates was apparent.

The result also suggests virulence of the entomopathogenic fungi is not always related to the original host or place of origin as none of the isolates tested are from the test insect based on the present study. The isolates PPRC-2, PPRC-14, PPRC-51, PPRC-GG and PPRC-HH, can be recommended as primary candidates for further research work in

order to develop a mycoinsecticide against *S. zeamais*.

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