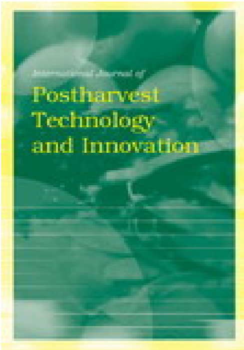

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Exploring the potential of using the entomopathogenic fungus *Beauveria bassiana* as a biocontrol agent for the maize weevil, *Sitophilus zeamais*

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Exploring the potential of using the entomopathogenic fungus *Beauveria bassiana* as a biocontrol agent for the maize weevil, *Sitophilus zeamais*

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Abstract: The use of entomopathogenic fungi as biological control agents has become a good method that effectively reduces post-harvest losses. The aim of this study was to evaluate the effect of *Beauveria bassiana* on the growth dynamic of *Sitophilus zeamais*, and the interaction between *B. bassiana* and *Aspergillus flavus* during storage. Four doses of *B. bassiana* were used, one combined with *A. flavus*. Three replicates of 250 g of treated maize and 20 unsexed adults of *S. zeamais* were added to each dose and assessed every 15 days, using a destructive assay. The mortality rate due to *B. bassiana* ranged between 53.7 ± 8.9% and 90.3 ± 4.0%. Aflatoxin contents in all treatments were lower than 0.25 µg/kg. Our study found an optimal recommended dose of 2 g of *B. bassiana* per kg of dry maize, and the activity of *S. zeamais* did not increase the aflatoxin content in stored maize.

Keywords: entomopathogenic fungi; *Beauveria bassiana*; biological control; maize weevil; *Sitophilus zeamais*; post-harvest losses; post-harvest technology; *Aspergillus flavus*; aflatoxin contamination.

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1 Introduction

Maize (*Zea mays L*) is the most widely produced staple crop in the world with 1.16 billion tonnes of production estimated in 2020. And production increasing an average annual rate of 3.41% (FAOSTAT, 2021). In Benin, maize is ranked first in national food system and remains the most consumed cereal, followed by rice and sorghum (Aminou, 2018). In tropical regions in Africa, the storage of maize without treatment may cause almost up to 40% loss of the grains (Lamboni and Hell, 2009; López-Castillo et al., 2018). These losses are mainly due to post-harvest insects and/or fungi, and cause a reduction in nutritional value and germination potential of the grains (Scheepens et al., 2011). Reducing post-harvest losses during storage of grains crops can strengthen food security in developing countries (Kumar and Kalita, 2017).

Post-harvest insects and grain storage conditions are considered as the main cause of grain loss (Gwinner et al., 1996; Chen et al., 2018). Among the insects, *Prostephanus truncatus* and *Sitophilus zeamais* are the main agents causing damage to maize stored with and without husk in Benin (Hell et al., 2008). Indeed, in rural areas where post-harvest management techniques are poorly developed, *S. zeamais* can cause up to 90% damage grains after five months of storage (Denning et al., 2009; Noosidum and Sangprajan, 2014). In addition to losses, post-harvest insects can convey mycotoxigenic fungi, such as *Aspergillus flavus*, to stored maize (Lamboni and Hell, 2009). Some strains of *A. flavus* may contaminate stored maize with aflatoxin B_1 (AfB_1) and aflatoxin B_2 (AfB_2) and make stored grains unsafe for consumption (Castegnaro and Pfohl-Leszkowicz, 2002). Optimising harvesting time, sorting, drying, good storage practices, use of controlled atmosphere, use of chemical pesticides, are shown to be good physical and chemical control measures that can mitigate the proliferation of post-harvest insects on stored maize (Arthur and Subramanyam, 2012; Karim et al., 2017; Sikirou et al., 2018). Biological control methods are based on the use of parasitoids, parasites and predators for post-harvest control of insects (Schöller et al., 2018; Adarkwah et al., 2019). Among the use of entomopathogenic fungi as biological control, *Beauveria spp.*, *Metarhizium spp.* and *Verticillium spp.* have shown promising results against most insects (Kassa et al., 2002; Humber, 2012; Samson et al., 2013).

Beauveria bassiana can infect its host by contact of by entering the body, leading to the death of the host (Halouane et al., 2007; Ortiz-Urquiza and Keyhani, 2016; Wang et al., 2021), but it is shown to be non-pathogenic to non-immuno compromised humans (Mascarin and Jaronski, 2016). A lot of authors have mentioned the protective effect for food commodities of using *B. bassiana* against post-harvest insects (Skinner et al., 2014; Rumbos and Athanassiou, 2017; Batta and Kavallieratos, 2018; Ak, 2019). Meikle et al. (2001) obtained satisfactory results mainly for the management of *Prostephanus truncatus* with *B. bassiana* on stored maize ears. *P. truncatus* densities were significantly lower in treatments that included conidia of *B. bassiana*. A combination of *B. bassiana* with wood ash showed efficacy for the control of *P. truncatus* (Smith et al., 2006). Under a laboratory trial where ten different species of entomopathogenic fungi were assessed for the control of the lesser grain borer *Rhyzoperta dominica*, *B. bassiana* showed the highest mortality rate of up to 65% (Musso et al., 2020).

A simple, effective and chemical-free method of protecting stored grain rom insect attack is required. The use of *B. bassiana* for the control of the post-harvest pest, *Sitophilus zeamais*, in stored maize was found effective. The main objective of this study was to assess the effect of *B. bassiana* on the development of *S. zeamais* in maize in the presence or not of toxigenic strain of *A. flavus*.

2 Material and methods

2.1 Experimental procedure

The trial was conducted from February to May 2019 at the International Institute of Tropical Agriculture in Benin. *Sitophilus zeamais* was collected from infested maize obtained from a Dantokpa market and reared in glass jars under laboratory condition of 30 ± 2°C and 85 ± 8 relative humidity. The *Beauveria bassiana* strain Bb 11 5653 and a toxigenic strain of *Aspergillus flavus* were obtained from the International Institute for Tropical Agriculture. The strain of *A. flavus* were inoculated on potato dextrose agar (PDA), incubated at ambient temperature under aseptic condition and dried. The upper layer was collected as a powder and stored for further use. The maize (variety TZPB SR-W) was locally purchased, dried, sieved, sorted to remove impurities and then stored at 4°C for two weeks to kill any biological organism present.

To differentiate the treatments, maize grains, *B. bassiana* and/or *A. flavus* were carefully mixed in a plastic container using an Enox spatula until the powders seemed to be evenly distributed over maize grains. Different treatments were prepared: T0 = 0 g of *B. bassiana*/kg of maize (control); T1 = 0.4 g of *B. bassiana*/kg; T2 = 2 g of *B. bassiana*/kg; T3 = 4 g of *B. bassiana*/kg and T4 = (2 g of *B. bassiana* + 2 g of *A. flavus*)/kg of maize kernels. Then 250 g of the mixture was transferred into one-litre glass jar. Later, 20 unsexed newly emerged adults of *S. zeamais* were randomly selected and added to the contents of each glass jar. The glass jar was closed with a lid fitted with a metal of 150 mm mesh to allow insect respiration but prevent to escape. All treatments were replicated three times for the seven sampling periods and stored on racks in the laboratory' storage room under ambient temperature (30 ± 2°C) and relative humidity (85 ± 8). To collect data, a destructive method was used. For each stored period, all glass jars were removed and assessed at 15, 30, 45, 60, 75, 90 and 105 days of storage. The temperature and the relative humidity of the room were recorded during the entire storage period and presented in Figure A1.

2.2 Assessment of moisture content and water activity of maize

The moisture content was determined using the oven-drying method according to the ISO 712:1979. From each glass jar, three replicates of 10 ± 1 g of maize grains were removed, milled and transferred into a metal container, weighed (Wi), dried for 2 h 15 min at 130°C, reweighed (Wd) and the moisture content (Mc) calculated as the percentage of the ratio of the difference in weight according to the formula Mc = 100[(Wi – Wd) / Wi]. The water activity (a_w) was measured with a thermo-hygrometer (Rotronic Hygrolab 2, 8303 Bassersdorf) following the method described by Anihouvi et al. (2006). The maize grains (subsample of 5 ± 1 g) were removed, milled, transferred in a plastic dish and placed into the thermo-hygrometer. A few minutes later, the a_w value is shown on a digital screen of the thermo-hygrometer. The measurement was duplicated and the average then used.

2.3 Assessment of mortality rate of S. zeamais due to B. bassiana during storage

At each assessment date, the content of a glass jar is sieved to separate maize grains from insects. Then dead *S. zeamais* and living *S. zeamais* were counted and the mortality rate was calculated. To assess insect' mortality due to the *B. bassiana* the method of Meikle et al. (2001) was used. From all treatments involving *B. bassiana*, each dead *S. zaemais* is washed with sterile distilled water with 10% sodium hypochlorite, rinsed three times in sterile distilled water, both to remove any surface contaminants, and the insect is kept separately in Petri dishes. All insects were then plated on PDA, Lab M Limited 1 Quest Park, Moss Hall Road, Heywood, Lancashier BL9 7JJ, UK amended with few droplets of streptomycin to limit bacterial growth. The plates were incubated under aseptic condition at ambient temperature for maximum six days. From the 3rd day, the growing fungus was then identified under microscope and recorded either as *B. bassiana* or not. Later, the mortality rate due to *B. bassiana* was calculated.

2.4 Assessment of grain damage and grain loss

Grain damage and grain loss were evaluated using the count and weigh method described by Boxall (2002). In three replicates, 1,000 randomly selected grains were taken, separated into damaged and undamaged grains, counted separately, weighed and then the grain damage and the grain weight loss were calculated according to the following formula: damage (%) = 100 × [Nd / (Nu + Nd)] and weight loss (%) = 100 × [(Wu × Nd) – (Wd × Nu)] / [Wu × (Nd + Nu)], where Nd is the number of damaged grains; Nu, the number of undamaged grains; Wd, the weight of damaged grains; and Wu, the weight of undamaged grains.

2.5 Aflatoxins contents of stored maize

All glass jars of the treatment supplemented with spores of toxigenic *A. flavus* were assessed for aflatoxin quantification to evaluate the antagonism effect of *B. bassiana* and *A. flavus* for the control of *S. zeamais*. For data assessment, three subsamples (25 g) of maize were taken and stored at 4°C until aflatoxin quantification. The samples were then sent to the Central Laboratory of Food Security control for aflatoxins B_1 (AfB_1) and B_2 (AfB_2) analysis using high performance liquid chromatography (HPLC) (ISO 16050:2003, 2003).

2.6 Statistical analysis

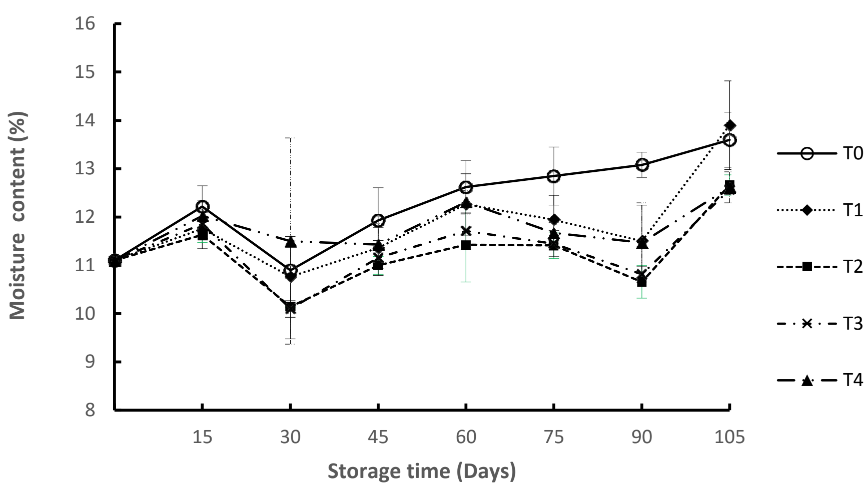
Collected data were subjected to one way – ANOVA with Box and Cox transformation for all values except aflatoxins contents, using Minitab version 17.1.0. Tukey test at 5% was used for pairwise comparison of means.

3 Results

3.1 Changes in moisture content and water activity of stored maize

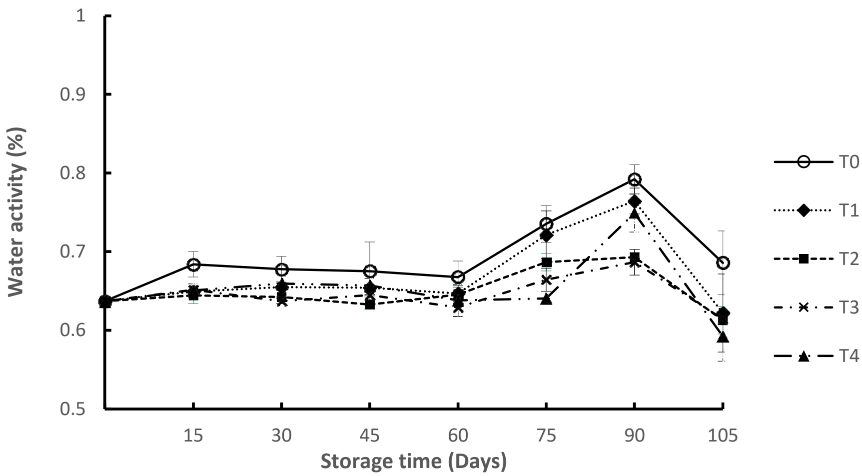
The moisture content of maize at the beginning of storage was 11.10% (Figure 1). During storage, and within all treatments, this value varied from 10.10% (T3, day 30) to 13.90% (T1, day 105). There were significant differences in moisture content between treatments on day 75 (P = 0.011), day 90 (P = 0.002) and day 105 (P = 0.011).

Figure 1 Mean moisture content (± SE) of maize during storage (see online version for colours)



The water activity was 0.64 at the beginning of storage (Figure 2). This value varied during storage to reach a maximum of 0.79 (T0, day 90) and a minimum of 0.59 (T4, day 105). There were significant difference in water activity between treatments on day 15 ($P = 0.005$), day 30 ($P = 0.001$), day 45 ($P = 0.049$), day 75 ($P = 0.001$) and day 90 ($P = 0.0001$).

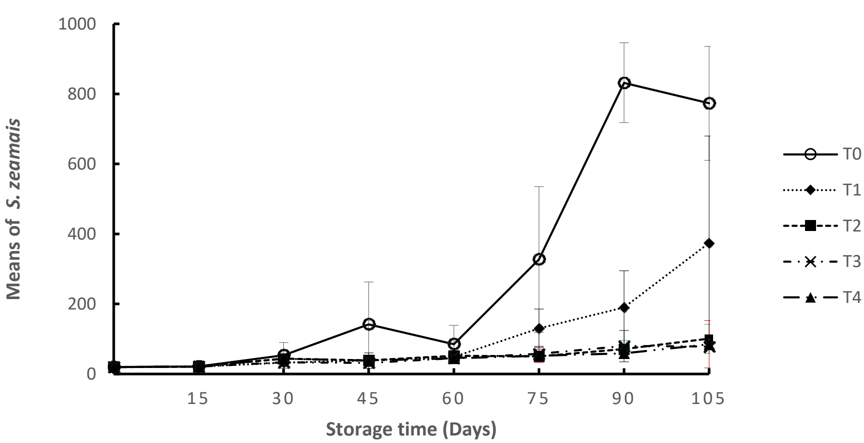
Figure 2 Mean water activity (± SE) of maize during storage (see online version for colours)



3.2 Growth dynamic of *S. zeamais*

During the storage period, the number of *S. zeamais* per jar increased to reach a maximum of 773 ± 162.75 on T0 and 374.33 ± 305.64 on T1 after 105 days storage. The number of *S. zeamais* remained lower than 100 in T2, T3 and T4 throughout the 105 days storage (Figure 3). A significant difference between the number of *S. zeamais* on treatments was noticed from the 45 days of storage ($P = 0.034$).

Figure 3 Mean number (± SE) of *S. zeamais* during storage



3.3 Mean mortality rate of S. zeamais

The mortality rates of *S. zeamais* during storage are shown in Figure 4. The control showed a maximum mortality rate of 28.14 ± 25.12% at day 60 and a minimum rate of 5.05 ± 2.24% at day 90. For all other treatments, the minimum rate recorded was 26.73 ± 23.76% (T1, day 90). ANOVA showed a significant difference between the mortality rate of treatments amended with *B. bassiana* and or *A. flavus* from 60, 75 and 90 days of storage with P = 0.005, P = 0.0001 and P = 0.014, respectively.

Figure 4 Mean mortality rate (± SE) of *S. zeamais* during storage (see online version for colours)

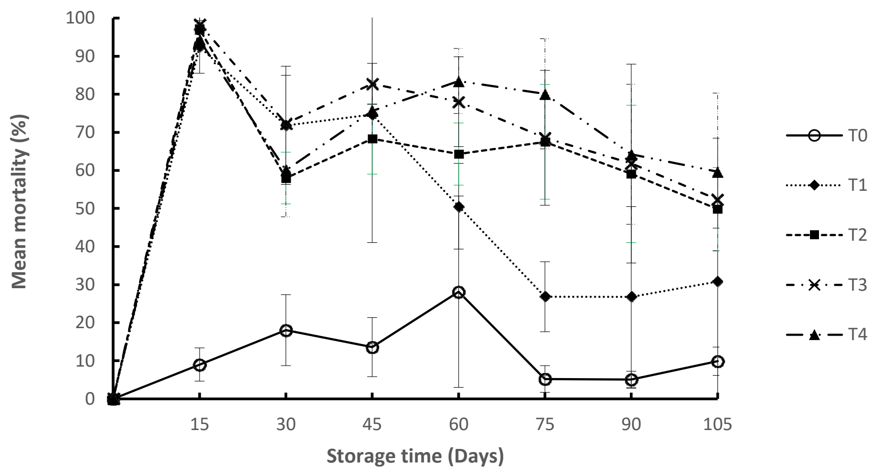
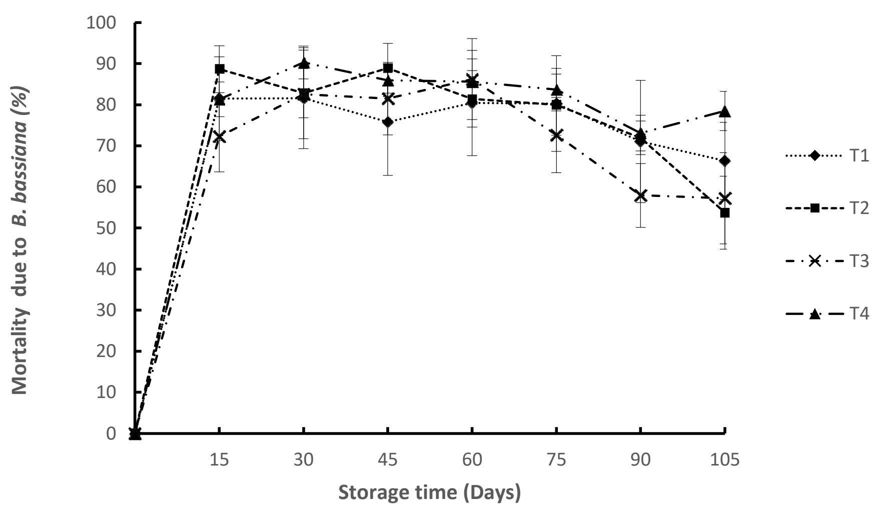


Figure 5 Mean mortality rate (± SE) of *S. zeamais* due to *B. bassiana*



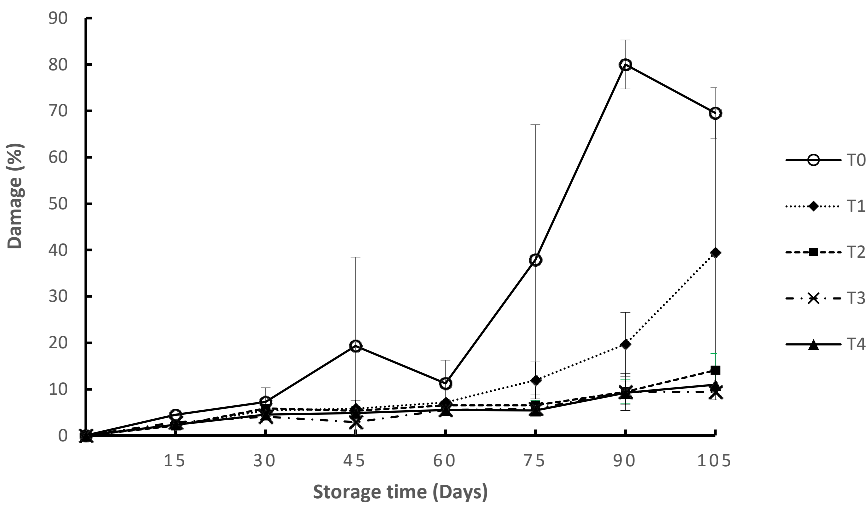
3.4 Mean mortality rate of S. zeamais due to B. bassiana

The mean mortality rates of *S. zeamais* due to *B. bassiana* during the storage are shown in Figure 5. The minimum recorded was 53.73 ± 8.89% (T2, day 105) and the maximum was 90.29 ± 3.97% (T4, day 30). ANOVA showed a significant difference between these rates only on 105 day 105 of storage (P = 0.036).

3.5 Grain damage and weight loss

Figures 6 and 7 showed the damage and losses caused by *S. zeamais* to maize during storage, respectively. The damage on control increased from 4.47 ± 0.15% on day 15 to 69.57 ± 5.44% on day 105 (Figure 6). For all others treatments, the damage had the same increasing pattern and significant differences were observed within treatments from day 90 (P = 0.001) to day 105 (P = 0.031).

Figure 6 Mean damage (± SE) caused by *S. zeamais* during storage (see online version for colours)



The weight loss caused by *S. zeamais* on the control increased from 2.47 ± 0.16% (day 15) to 26.35 ± 5.81% (day 90) with a decrease to 15.41 ± 1.65% (day 105) (Figure 7). For all other treatments, maize weight losses started lower than 1% to a maximum of 3.32 ± 0.55% except a maximum of 9.17 ± 6.25% for T1. ANOVA showed that there were significant differences between losses at day 75 (P = 0.0001), day 90 (P = 0.0001) and day 105 (P = 0.007).

3.6 Aflatoxins contents

AfB_1 and AfB_2 contents in maize amended with *A. flavus* were less than 0.25 µg/kg, compared to 5 µg/kg which is the maximum tolerated limit by European Union Regulation 1881/2006. The results of aflatoxins assay are presented in Table 1.

Figure 7 Weight loss (± SE) caused by *S. zeamais* during storage

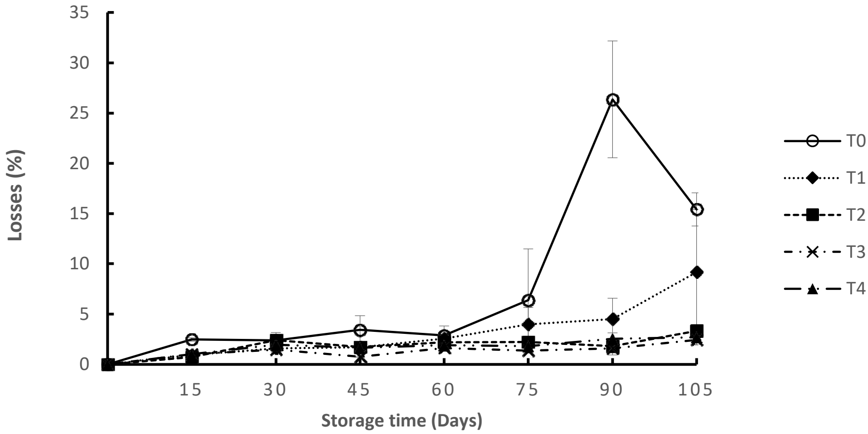


Table 1 Aflatoxins contents (µg/kg) on maize amended with *A. flavus* (T4) compared to T0 (control)

Days	*15*	*30*	*45*	*60*	*75*	*90*	*105*
			AfB_1 (µg/kg)				
Standard			<5 µg/kg				
T0	<0.25	-	-	-	-	-	<0.25
T4	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
			AfB_2 (µg/kg)				
Standard			<5 µg/kg				
T0	<0.25	-	-	-	-	-	<0.25
T4	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25

Note: T0: control; T4: maize + *B. bassiana* + *A. flavus*.

4 Discussion

4.1 Effect of B. bassiana on the growth of S. zeamais

This study demonstrated a significant reduction of number of *Sitophilus zeamais*, grain damage, grain weight loss and a high mortality rate of *S. zeamais* when maize was stored mixed with *B. bassiana*. According to Tefera et al. (2010), cereals dried at 12–14% water content are not prone to fungi growth, but are still good for insects' infestation. The water content of 11.10% observed at the beginning of storage of the maize may ensure that only fungal agents introduced into the storage system can have effect on the development of *S. zeamais*. The optimal temperature for *S. zeamais* is generally between 27 and 32°C with relative humidity being around 70% (Gwinner et al., 1996; Ojo and Omoloye, 2012, 2016). These were similar during our study giving to *S. zeamais* good climatic conditions for its development.

The development of *S. zeamais* was relatively controlled by the use of the entomopathogenic fungus *B. bassiana*. It has reduced the multiplication of *S. zeamais* and increased its mortality. The mobility of *S. zeamais* may spread fungi in maize stores and may increase the proliferation of the entomopathogenic fungus. Mul et al. (2009) and Kaoud (2010) reported that after the death of the insect, the entomopathogenic fungus could grow out and produce more spores, increasing contamination and mortality for other mobile insects. Naturally occurring entomopathogenic fungi on storage insect pests were reported on *S. zeamais*, *Tribolium spp.*, *Carpophilus spp.* and *Rhyzopertha dominica* (Oduor et al., 2000; Er et al., 2016).

Comparable to our results, Adane et al. (1996) demonstrated the ability of the conidia of *B. bassiana* to infect *S. zeamais* and cause mortality of nearly 88% in eight days. Several other authors have confirmed the effectiveness of *B. bassiana* in *S. zeamais* induced contamination and mortality tests (Meikle et al., 2001; Barra et al., 2013; Mbata et al., 2018). The efficacy of *B. bassiana* has also been proven on other *Sitophilus* species such as *S. oryzea* for its control in rice stocks (Kavallieratos et al., 2014; Er et al., 2018). *Prostephanus truncatus* (Meikle et al., 2001, 2002; Acheampong et al., 2016) and *Tribolium spp*. (Barra et al., 2013; Athanassiou et al., 2016) are other maize post-harvest insects that have been effectively controlled by *B. bassiana* with high mortality.

Assessing the mortality rate of *S. zeamais* due to *B. bassiana*, the results ranged from 53.73 ± 8.89% to 90.29 ± 3.97%, showing that *B. bassiana* is responsible for more than half of *S. zeamais*' deaths. These results are also similar to those of Teshome and Tefera (2009) who found a mortality rate ranging from 25 to 95.5%. Later, Rondelli et al. (2012) recorded a mortality rate between 51.3 to 68%. However, Oduor et al. (2000) in their study on the natural prevalence of *B. bassiana* on predatory insects of maize stocks in three agro-ecological zones of Kenya, recorded a prevalence rate of 0.08 to 0.94% especially on *S. zeamais*. This demonstrates the low prevalence rate of *B. bassiana* naturally occurring on post-harvest insects and of maize stocks. Therefore, in a biological control objective using an entomopathogenic fungi, an additional contribution of *B. bassiana* is necessary for an optimal control and an effective fight against *S. zeamais*.

A reduction in damage and losses caused by *S. zeamais* in maize stocks was noticed with significant different between treatments on 90 and 75 days, respectively, in T2 (2 g/kg) and T3 (4 g/kg) compared to control. An augmented spores of *B. bassiana* in a control environment is believed to reduce the damage and losses caused by *S. zeamais* to stored maize proportionally to the reduction in *S. zeamais*' population. *B. bassiana* would therefore have modified the feeding behaviour of *S. zeamais*, inducing high mortality. Storm et al. (2016) in their study of induction of palatability by Kaolin in the presence of *B. bassiana*, recorded an increase of mortality rate of *Sitophilus granarius* from 46% to 88% and from 81% to 99%, 7 and 14 days after treatment, respectively. Similarly, Tefera and Pringle (2003) showed a reduction in feeding behaviour three days after the inoculation with spores of *B. bassiana* of larva of *Chilo partellus* (pyralidae, larva stage 2 very active on maize).

In general, the study demonstrated that the dose of 2 g/kg would be the optimum for the control of *S. zeamais* by *B. bassiana*.

4.2 Effect of B. bassiana on the growth of S. zeamais in the presence of A. flavus

In our study, we added 2 g of spores of *A. flavus* to maize in T4, in order to simulate a strong contamination of the maize by spores of a toxigenic strain of *A. flavus*. The results showed a significant difference on 105 days of storage and at the same dose of *B. bassiana*, the mortality rate of *S. zeamais* due to *B. bassiana* increased in the presence of *A. flavus*. Interaction between microorganisms and members of other species (Liu et al., 2013) or even other genera (Perez et al., 2011) have been reported. Moreover, combination of *B. bassiana* with other physical compounds that increase the effectiveness of the entomopathogenic fungus have been demonstrated. Smith et al. (2006) used wood ash against *Prostephanus truncatus*; Lord (2001), Akbar et al. (2004) and Wakil et al. (2012) used diatomaceous earth against *Rhyzopertha dominica* and *Tribolium spp.* whereas Storm et al. (2016) added Kaolin to *B. bassiana* against post-harvest insects.

To assess the impact of the simultaneous presence of *B. bassiana* and *A. flavus* on the possible production of mycotoxin on stored maize, we carried out the aflatoxin B_1 (AfB_1) and B_2 (AfB_2) assay. The aflatoxin levels were below 0.25 μg/kg throughout storage, for both AfB_1 and AfB_2. These contents comply with the requirements of EU regulation 1881/2006 fixing a maximum content of 5 μg/kg "for maize intended to be subjected to sorting treatment or other physical methods before human consumption or use as food ingredient." The recorded water activity between 0.59 and 0.76 did not allow spores of *A. flavus* to grow to a possible formation of aflatoxins on stored maize.

5 Conclusions

In order to control the development of *Sitophylus zeamais* in stored maize, the efficacy of several doses of *B. bassiana* was tested. At a lower dose of 0.4 g/kg, the damage and weight losses caused by *S. zeamais* were reduced and the mortality rates were above 50% for *S. zeamais* from 90 days of storage. At the doses of 2 g/kg and 4 g/kg, the mortality rates were above 50% from 75 days of storage onwards. Thus, among the three doses, the optimal recommended dose for an effective control of *S. zeamais* in stored maize could be 2 g/kg. Also, it has been observed that the combination of *B. bassiana* and *A. flavus* lead to a higher mortality rate for *S. zeamais* with no production of aflatoxins in stored maize.

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Appendix

Figure A1 Temperature and relative humidity in the room during the storage period (see online version for colours)