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Insecticidal efficacy of *beauveria bassiana*, diatomaceous earth and fenitrothion against *rhyzopertha dominica* and *tribolium castaneum* on stored wheat

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ABSTRACT

Bioassays were conducted in stored grains to evaluate the combined use of the entomopathogenic fungus *Beauveria bassiana*, diatomaceous earth (DE) and fenitrothion against adults of *Rhyzopertha dominica* and *Tribolium castaneum*. The insecticide agents were applied to wheat as follows: the fungus at 1×10^8 conidia/kg of wheat, DE at 3 g/kg of wheat and fenitrothion at a rate of 0.15 ml/kg of wheat (25% of field dose rate). Surviving insects and progeny production were monitored at thirty-day intervals until four months. For both insect species, significantly less progeny was produced on wheat treated with fenitrothion and DE + *B. bassiana* formulations in comparison with controls. The effect of insecticides on the grain damage, germination power of wheat and bulk density was also evaluated. After four months, *B. bassiana* mixed with DE reduced the damaged insect grains by 50% in comparison with both fenitrothion and control treatments. Trials using DE caused a reduction in bulk density while there were no significant differences for germination testing between treatments.

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Diatomaceous earth; *Beauveria bassiana*; fenitrothion; wheat grain; stored-product beetles

1. Introduction

Insects are a major cause of postharvest grain losses. They not only consume the grain but also contaminate it with insect bodies, feces, and foul smelling metabolic products. Insect activity can also promote the growth of a variety of microflora, some of which pose serious health hazards to humans and livestock, particularly those producing mycotoxins (Dawson et al. 2004; Richard et al. 2007; Houssou et al. 2009). Coleoptera species, especially the grain beetles are often the most abundant and destructive pests (Lord et al. 2007). They include the lesser grain borer, *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) and the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *R. dominica* is considered a primary pest, because insects can easily infest sound seeds, enabling other species (the secondary pests, which are not capable of breeding on intact grains) to cause additional damage. Nevertheless, this trend is completely reversible; Trematerra et al. (2015) found that kernels that were previously infested by primary colonizers, such as *R. dominica* and *Sitophilus zeamais*, were more prone to be selected by secondary colonizers such as *T. castaneum* adults, and kernels that were previously infested by *T. castaneum* were more susceptible to be selected by *S. zeamais* adults. *R. dominica* larvae develop exclusively in the internal part of the grain, where they are not greatly affected by the presence of contact insecticides on the outside of the kernel. Moreover, this species has developed a considerable level of resistance to traditional grain protectants and fumigants (Arthur 1996; Athanassiou et al. 2008a). *T. castaneum* can be a major pest in structures used for the processing and storage of grain-based products. This secondary pest is a cosmopolitan insect whose adults and larvae cause economic damage in a wide range of commodities including grain, flour, peas, beans, cacao, nuts, dried fruits, and spices, but milled grain products appear to be their preferred food (Campbell and Runnion 2003). Among the potential pest species of the genus *Tribolium*, *T. castaneum* and the confused flour beetle, *T. confusum*, are perhaps the most problematic due to their prevalence and ability to survive most control measures (Lord et al. 2007; Athanassiou et al. 2013).

Currently, the use of chemical insecticides is the main solution to prevent the damage caused by stored product pests, inducing ecotoxicity problems and the occurrence of resistance within insect populations (Wang et al. 2008; Saeed et al. 2017). The process of resistance development can be effectively delayed by using integrated pest management (IPM) strategies that mainly focuses upon the use of biological control methods and the application of safer and selective insecticides (Wakil et al. 2012). Therefore, the exclusive use of fumigants or residual insecticides to control

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storage insect pests should be discouraged. Such concerns have led to the development of nontoxic alternatives to traditional insecticides for the control of storage pests that infest food commodities (Sakenin Chelav and Khashaveh 2014). The mycopesticides have shown considerable potential for the management of insects and its pathogenicity has been used worldwide to control various coleopteran pests in grain storage and processing environments (Khan and Selman 1984, 1987, 1988; Rodrigues and Pratissoli 1990; Padín et al. 1997). Many of these important pests have proven to be susceptible targets for the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales). *B. bassiana* has previously been shown to provide moderate to high levels of control of coleopteran pests in stored grain including *R. dominica* (Moino et al. 1998; Rice and Cogburn 1999; Lord 2001; Vassilakos et al. 2006) and *T. castaneum* (Padín et al. 1997; Rice and Cogburn 1999; Akbar et al. 2004) in stored grain. DE are fossilized skeletorns of phytoplankton (diatoms), whose major constituent is amorphous silicon dioxide, that kill insects by abrading the cuticle and thus absorbing the epicuticular lipids causing water loss through dessication. The use of DE has been widely proposed as an alternative to the use of traditional contact insecticides in stored product protection (Arthur 2000; Athanassiou et al. 2005; Kavalieratos et al. 2007, 2015; Athanassiou et al. 2008a; Iatrou et al. 2010; Athanassiou et al. 2014, 2016). Entomopathogenic fungi and DE offer many options including a degree of efficacy comparable with chemical pesticides and compatibility with most of the other components of IPM strategies. According to several reports, the combined use of DE and *B. bassiana* has proven to have higher insecticidal effect than that it was exploited when fungal preparations were used alone (Lord 2001; Akbar et al. 2004; Kavallieratos et al. 2006). However, most of these studies were carried out under laboratory conditions (Dal Bello et al. 2006; Kavallieratos et al. 2006; Michalaki et al. 2006; Vassilakos et al. 2006; Athanassiou and Steenberg 2007; Athanassiou et al. 2007; Athanassiou et al. 2008b; Dal Bello et al. 2011). In this study, we examined the insecticide potential of the combination of *B. bassiana*, DE, and fenitrothion to protect stored grain from two major stored product beetle species, *R. dominica* and *T. castaneum*, under more real, practical conditions.

2. Material and methods

2.1. Test insects

Both *R. dominica* and *T. castaneum* adults come from well-established colonies, maintained at the Agricultural Faculty, University of La Plata, Argentina for fifteen years without exposure to insecticides. Experimental insects were cultured in 500 ml glass jars in a controlled temperature and humidity growth chamber (range 27 ± 2 °C, 60%–70% r.h., 12:12 light: dark). *R. dominica* was reared on whole wheat grain, and *T. castaneum* on white wheat flour, with 5% non-fat dried milk, 5% brewer's yeast and 5% wheat germ. All adults used in the assays were 2-week-old, unsexed insects.

2.2. Fungal culture

The *B. bassiana* (Bb) isolate used in the tests was obtained from stored grain in the Buenos Aires province, Argentina. This strain was originally isolated from an infected individual of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). Insect was held in a moist chamber consisting of a sterile Petri dish with a disc of filter paper moistened with sterile distilled water, and incubated at 25 °C. Daily checks were performed up to the seventh day. A filamentous fungus emerging from the insect and identifiable as the genus *Beauveria* was transferred to Petri dishes containing Sabouraud dextrose agar medium (SDA, Difco Laboratories, Detroit) supplemented with streptomycin sulfate (500 mg/l^{-1}, Sigma, St. Louis, MO, USA). Plates were incubated in total darkness at 25 °C and developing colonies were subcultured onto new SDA plates without antibiotic. From these cultures, monosporic isolates were prepared by arbitrarily selecting one colony forming unit (CFU) after plating a serially diluted conidia suspension of the isolate. Plates were incubated at the same temperature as before, promoting the development of a pure culture for subsequent identification of the species according to Humber (1997). *Beauveria bassiana* was identified by macromorphological aspects of the colonies, such as color, diameter, mycelial texture, and also for their micromorphological characteristics, as observed under an optical microscope. After identification at the species level the strain was maintained at the fungal collection of the Centro de Investigaciones de Fitopatología (CIDEFI), University of La Plata. To carry out the bioassays, the pathogen was further sub-cultured on SDA and incubated at 25 ± 2 °C in a growth chamber under a 12-h fluorescent plus near ultraviolet photoperiod to induce sporulation for 2 weeks. Conidia were harvested immediately prior to use, suspended in 0.01% Tween 80 in sterile distilled water, and vortexed for approximately 3 min. The conidial suspension was filtered through a 75 μm sieve to remove debris. The number of conidia were estimated with a Neubauer haemocytometer and adjusted to 1 × 10^8 conidia ml^{-1}. Conidial viability was confirmed by plating 100 μl of a 10^8 conidia suspension on quarter strength malt extract agar (MEA) and incubated at 25 °C in total darkness. After 24 h germination rate was determined. Conidial viability was always >95% for all tested suspensions.

2.3. Diatomaceous earth

DE came from seven deposits in Ingeniero Jacobacci (Río Negro, Argentina). DE blocks were reduced in size to 2–5 cm, ground, passed through mesh 18 (1 mm), and dried to constant weight at 110 °C to determine water content. DE passing through mesh 325 (43 mm) was collected and tapped density determined (Korunic et al. 1996). It is white in colour, has a tapped density 238 g l^{-1} and pH 8.4 with median particle size of 43 μm.

2.4. Insecticide

The insecticide used was fenitrothion emulsion ([O,O-Dimethyl-O-(3-methyl-4-nitrophenyl)- phosphorothioate]; Nufarm®, EC 100%) at a rate of 0.015 ml kg^{-1} of wheat. This volume is proportional to 25% of the labelled field dose rate (60 ml of EC per 1000 kg of wheat) for fenitrothion.

2.5. Germination assessment

The effect of fenitrothion on conidia germination was tested as follows. The culture medium SDA amended with a fenitrothion concentration of 0.012 ml l^{-1} (≈0.015 ml kg^{-1} of wheat) was poured into 5.5 cm plastic Petri dishes. *B. bassiana* conidia were suspended in distilled water containing 0.01 ml l^{-1} of Tween 80 (1×10^8 conidia ml^{-1}) and inoculated in the plates. After incubation for 24 h at 25 ± 2 °C in the dark, the percentage of spore germination (100 conidia for each of 3 replicates) was estimated under a microscope.

2.6. Bioassays

Bioassays were carried out on hard wheat (var. ACA 303) with moisture content of 14%, determined with Hygrometer Delver HD 1000 LC. Before used in the test, grains were frozen for 20 days at −18 °C to kill any residual insect infestation. To simulate the real storage conditions, the experiments were conducted in 80 plastic jars (50 cm height and 35.5 cm diameter) with wheat for 30, 60, 90 and 120 days. Four groups of 20 jars containing 2000 g of wheat (whole-kernel) each, in a randomized complete design were used. Each group consisted of four treatments: (1) insecticide at 25% of the labelled rate (F25), (2) Bb + DE, (3) Bb + DE + F25, and (4) untreated control (five replicates of each treatment). Twenty adults of both insect species were added to each jar which was later capped with a muslin cloth to facilitate ventilation and preventing insects from escape. The fungal suspension was sprayed at the rate of 1 ml kg^{-1} of wheat. This proportion is similar to that used in other studies on stored grain beetles (Lord 2001; Dal Bello et al. 2011). DE dose (3000 mg kg^{-1}) was selected considering previous work on a variety of DE of different origin and purity (Korunic 1997). The control was sprayed with tap water at the rate of 1 ml kg^{-1} of wheat grain. The insecticide, the fungal suspension and water were sprayed individually by using a Badger 100® Artist airbrush (Thayer and Chandler). The grain treated was allowed to dry before being placed in incubators set at 27 °C and 65% ± 5% RH for 2 days to equilibrate moisture (Kavallieratos et al. 2009). To assay DE, the dust was added to wheat in the jar. To ensure even distribution in the entire grain mass, the jars were shaken manually for 2 min. All experiment was conducted at 14 °C and 18% ± 5% RH. At each time period (30, 60, 90 and 120 days), twenty jars (five replicates of each treatment) were removed from the incubator. Each jar was stirred for 2 min and two samples of grain (100g each one) was removed. These two samples were mixed carefully and the number of live beetles, grain loss and germination power of wheat seeds were recorded. Later on, the entire set was discarded.

2.7. Grain loss

Damaged kernels were assessed from 5 samples of 200 g of wheat extracted monthly from each treatment and control. Damaged insect grains were manually separated, weighed and introduced to this formula for the calculation of percentage of weigh damaged kernels: % damaged insect grains = (weight of damaged kernels/total sample weight) × 100.

2.8. Bulk density and seed germination tests

The effect of DE concentration on bulk density (hectoliter mass) of wheat kernel was determined by use of the standard method (Schopper scale). The bulk density was determined 30 min after treatment and the measurement was repeated five times for each treatment and control.

The germination ability was evaluated on 25 healthy grains of wheat which were placed on Whatman N° 3 filter paper in a 90-mm diameter Petri-dish saturated with 5.5 ml of distilled water. The covers of the Petri dishes were closed and they were kept at 25 °C in the dark in growth cabinets for 72 h. The percent of germinated seeds after 72 h was referred to as germination rate. Five replicates were prepared for each treatment and control at intervals of 30 days.

2.9. Statistical analysis

The data were subjected to ANOVA with Statgraphics Plus software (Manugistics, Rockville, MD) and means were compared by Tukey's test. For comparing 30–120 days treatments Student's *t* test was used.

3. Results

3.1. Germination assessment

Compatibility tests of F25 with *B. bassiana* demonstrated that formulation did not inhibit the germination of conidia (no effect at 0.012 ml l^{-1}; data not shown). Average germination percentage of the fungal isolate was 98% after 24 h of incubation.

3.2. Bioassays

The *T. castaneum* progeny was significantly different between treatments at all storage period assayed. Adult population of red flour beetles was significantly higher in untreated wheat grains than in the treated grains all through the 4 months ($p = 0.0002$; $p = 0.0037$; $p < 0.0001$ and $p < 0.0001$ for the first, second, third and fourth month, respectively). From the second to the fourth month no insects were found in the treatments with Bb + DE, but in those with only the insecticide, live insects were registered until 90 days. Comparing the first and the fourth month in each treatment there were no statistically significant differences (Table 1). For *R. dominica*, during the initial 2 months there were significant differences ($p = 0.0216$ for the first month and $p = 0.0135$ for the second month) in progeny between the control and the mix of F25 + Bb + DE. Over time, all treatments were significantly different compared with the untreated grain ($p = 0.0125$ and $p < 0.0001$ for the third and fourth month, respectively). Comparing the first and the fourth month, in Bb + DE treatments there were no statistically significant differences for the number of live adults of *R. dominica*, while they increased significantly in F25 ($p = 0.0209$) trials and control ($p < 0.0001$) (Table 2).

3.3. Grain loss

The ANOVA showed significant differences between treatments at 30 days ($p = 0.0140$), 90 days ($p = 0.0236$) and 120 days ($p < 0.0001$) for grain loss. Three months after treatments, biological insecticides (Bb + DE) caused a significant reduction in the loss of grains compared to the control. Interestingly, after four months of storage, treatments Bb + DE and Bb + DE + F25 produced a significant reduction in grain damage compared with the control as well as chemical insecticide alone ($p < 0.0001$). Considering the 30–120 days exposure interval, significant differences were noted in the control ($p < 0.0001$). This situation was also found in containers treated with F25 alone ($p < 0.0350$). The treatments Bb + DE and Bb + DE + F25 produced less grain loss than the control and also than F25 (Table 3).

3.4. Bulk density and seed germination tests

For test weight, there were no significant differences between F25 + DE + Bb and DE + Bb but both values were significantly lower than those of the control and

Table 1. Number (mean ± SEM) of live *Tribolium castaneum* adults per sample (200 g of wheat grain) treated with various insecticides at an exposure time of 30, 60, 90 and 120 days.

Treatments	30 days[a]	60 days	90 days	120 days
Control[b]	3.0 ± 0.54 a A	2.4 ± 0.75 a	2.2 ± 0.49 a	4.0 ± 0.55 a A
Fenitrothion 25	0.6 ± 0.40 b A	1.0 ± 0.45 ab	0.0 ± 0.00 b	0.0 ± 0.00 b A
Bb + DE	0.4 ± 0.25 b A	0.0 ± 0.00 b	0.0 ± 0.00 b	0.0 ± 0.00 b A
Bb + DE+ F25	0.2 ± 0.20 b A	0.0 ± 0.00 b	0.0 ± 0.00 b	0.0 ± 0.00 b A

[a] Means within each column followed by the same lowercase letters are not significantly different ($\alpha = 0.05$; Tukey's test).
[b] Means in the same row followed by the same uppercase letter are not significantly different ($p = 0.05$; Student's *t* test).

Table 2. Number (mean ± SEM) of live *Rhyzopertha dominica* adults per sample (200 g of wheat grain) treated with various insecticides at an exposure time of 30, 60, 90 and 120 days.

Treatments	30 days[a]	60 days	90 days	120 days
Control[b]	1.4 ± 0.40 a A	1.6 ± 0.40 a	16.6 ± 3.28 a	23.6 ± 2.32 a B
Fenitrothion 25	0.6 ± 0.40 ab A	0.6 ± 0.40 ab	1.2 ± 1.23 b	3.2 ± 1.59 b B
Bb + DE	0.2 ± 0.20 ab A	0.4 ± 0.25 ab	1.8 ± 0.74 b	0.0 ± 0.00 b A
Bb + DE + F25	0.0 ± 0.00 b A	0.0 ± 0.00 b	1.6 ± 0.68 b	0.6 ± 0.40 b A

[a] Means within each column followed by the same lowercase letters are not significantly different ($\alpha = 0.05$; Tukey's test).
[b] Means in the same row followed by the same uppercase letter are not significantly different ($p = 0.05$; Student's *t* test).

Table 3. Percentage (mean ± SEM) of weight loss of grains treated with various insecticides at an exposure time of 30, 60, 90 and 120 days.

Treatments	30 days[a]	60 days	90 days	120 days
Control[b]	0.112 ± 0.029 ab A	0.184 ± 0.028 a	0.416 ± 0.101 a	0.959 ± 0.039 a B
Fenitrothion 25	0.248 ± 0.061 b A	0.155 ± 0.046 a	0.219 ± 0.093 ab	0.566 ± 0.110 b B
Bb + DE	0.074 ± 0.039 a A	0.120 ± 0.015 a	0.092 ± 0.041 b	0.059 ± 0.020 c A
Bb + DE + F25	0.056 ± 0.012 a A	0.102 ± 0.023 a	0.112 ± 0.027 b	0.021 ± 0.012 c A

[a] Means within each column followed by the same lowercase letters are not significantly different ($\alpha = 0.05$; Tukey's test).
[b] Means in the same row followed by the same uppercase letter are not significantly different ($p = 0.05$; Student's *t* test).

Table 4. Effect of 3000 ppm of diatomaceous earth on wheat bulk density.

Treatments	Bulk density (k/hl) ± SEM[a]
Bb + DE + F25	70.74 ± 0.428 a
Bb + DE	70.97 ± 0.390 a
F25	74.80 ± 0.398 b
Control	75.93 ± 0.531 c

[a]Means within each column followed by the same letters are not significantly different ($\alpha = 0.05$; Tukey's test).

Table 5. Percentage (mean ± SEM) of germinated grains treated with various insecticides at an exposure time of 30, 60, 90 and 120 days.

Treatments	30 days[a]	60 days	90 days	120 days
Control	97.6 ± 3.07 a	98.4 ± 2.83 a	96.8 ± 0.84 a	95.6 ± 1.64 a
F25	98.4 ± 2.90 a	96.6 ± 0.68 a	96.4 ± 1.63 a	96.8 ± 0.84 a
Bb + DE	98.0 ± 1.91 a	97.6 ± 2.11 a	97.6 ± 0.68 a	97.4 ± 2.24 a
Bb + DE + F25	97.6 ± 2.83 a	98.0 ± 1.91 a	98.0 ± 1.91 a	97.2 ± 3.77 a

[a] Means within each column followed by the same letters are not significantly different ($\alpha = 0.05$; Tukey's test).

F25 treatments ($p < 0.0001$) (Table 4). The germination rate data was monthly evaluated and analyzed by ANOVA, with no significant differences between the control and treatments in any period (Table 5).

4. Discussion

The current study showed that biological insecticides confer acceptable control levels in stores bigger than those usually used in laboratory tests, being a more realistic situation resembling the usual field conditions, and protect grains from insect damage even better than a chemical insecticide used at an IPM-compatible dose.

The knowledge of the compatibility between entomopathogenic fungi and pesticides used in crop protection is essential in IPM programs (Todorova et al. 1998). Many experiments have been carried out to detect pesticide side effects on *B. bassiana* strains with variable results depending on the isolates, active ingredients and doses (Gardner and Storey 1995; De Oliveira et al. 2003; De Oliveira and Oliveira Janeiro Neves 2004; Alizadeh et al. 2007). According to Neves at al. (2001), the *in vitro* compatibility tests should consider inhibition or not of conidia germination when mixed with the formulations since this is an important factor at field conditions. The authors pointed out that the use of insecticides, in the recommended formulations and other concentrations tested, in most cases, had no negative effect on conidia germination, conidia production and vegetative growth of *B. bassiana*. In contrast, studies *in vitro* and under field conditions indicated the inhibition of *B. bassiana* germination, vegetative growth and sporulation by fenitrothion even at half of the recommended dose (Mejía et al. 2000; Tamai et al. 2002; Cazorla and Moreno 2010). On this basis, we tested fenitrothion at 25% of field dose rate which had no negative effect on the conidia germination of *B. bassiana*. The wide variability in sensitivity of *B. bassiana* isolates and other fungal species to chemical insecticides, reported in several studies, could be due to diverse factors including the intraspecific genetic variability within fungal species, not only in their response to synthetic chemicals, but also to various physical and biological parameters (Olmert and Kenneth 1974; Paccola-Meirelles and Azevedo 1990; Bello and Paccola-Meirelles 1998; Todorova et al. 1998; Tamai et al. 2002). Moreover, as pointed out by Tamai et al. (2002), commercial formulations made with the same active ingredient by different manufacturers may have different biological effects. Although many studies on the pesticide effects on fungal physiology are already available, conclusive information on the entomopathogen metabolic response to this challenge is rather scarce. In this regard, Forlani et al. (2014) showed no metabolic detrimental effects on *B. bassiana*, measured by antioxidant enzymes and lipid peroxidation, after fungal combination with deltamethrin at the usual doses applied in the field.

Arthur (2003) proposed that a possible alternative to using high DE doses could be the use of DE in combination with other, IPM-compatible control methods. There is strong evidence that DE can be successfully used in combination with entomopathogenic fungi (Lord 2001; Kavallieratos et al. 2006; Michalaki et al. 2006; Vassilakos et al. 2006; Athanassiou et al. 2008b), or low doses of insecticides (Athanassiou 2006; Athanassiou et al. 2007). Lord (2001) found that DE have a synergistic interaction with *B. bassiana* against some stored grain pests, while showing no negative effects on germination of the fungus. Akbar et al. (2004) reported that DE enhances the efficacy of *B. bassiana* against larval *T. castaneum*, at least in part by damaging the insect cuticle, thus increasing conidial attachment and making nutrients more available to conidia for their germination. The combined use of DE with *B. bassiana* was effective for control of *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae), and the median lethal time of adults was significantly lower than with DE or *B. bassiana* alone (Dal Bello et al. 2006; Vassilakos et al. 2006). Similarly, Lord et al. (2007) reported that DE and *B. bassiana* were synergistic against *R. dominica* and *Oryzaephilus surinamensis* L. (Coleoptera: Silvanidae) in laboratory tests. Athanassiou and Steenberg (2007) also demonstrated that simultaneous presence of *B. bassiana* and DE increased mortality of *Sitophilus granarius* L. (Coleoptera: Curculionidae).

The current study suggests that the optimal population control can be obtained with the combination of DE + Bb. This insecticidal effect did not increase with F25, demonstrating that the potential insecticide of both biological agents is comparable to that of some chemicals. In addition, several studies stated that *B. bassiana* is non-toxic to humans and other vertebrates as well as DE formulations have low mammalian

toxicity, rendering them a potential and safe method for pest control (Arooni-Hesari et al. 2015). Also, we found no significant differences of progeny production recorded between the first and fourth month for all treatments; thus, the insecticidal effect achieved early and maintained during several months might be especially useful to pest control in grains that are expected to be stored for long periods. We concluded that additional investigations are needed to elucidate the optimal conditions for best insecticidal efficacy of the combined chemical-biological treatments with the aim of their implementation into the strategies of environmentally acceptable control of *R. dominica*, *T. castaneum* and other economically important stored product insect pests.

Disclosure statement

No potential conflict of interest was reported by the authors.

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