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Comparative Evaluation of Four Agrochemicals against *Cryptolestes ferrugineus* and *Tribolium castaneum* along with Subsequent Infection Inhibition of Aflatoxigenic *Aspergillus* spp. in Stored Wheat

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ABSTRACT

In the present study, we evaluated toxicity of selected biorational insecticides (spinosad, thiamethoxam, indoxacarb and imidacloprid) against Pakistani strains of *Cryptolestes ferrugineus* (rusty grain beetle) and *Tribolium castaneum* (red flour beetle), and tested whether insects' mortality could control fungal contamination in stored grains or not? Bioassays were performed on wheat grains artificially infested with *C. ferrugineus* or *T. castaneum* along with spores of *Aspergillus flavus* or *A. parasiticus*. The results revealed that all the tested insecticides proved to be more toxic against *C. ferrugineus* as compared to *T. castaneum*. *C. ferrugineus* showed complete mortality at the highest concentrations of spinosad (1 ppm) and thiamethoxam (4 ppm), while >95% mortality was recorded at the highest concentrations of imidacloprid (4 ppm) and indoxacarb (4 ppm). In the case of *T. castaneum*, about 51%, 98%, 54% and 62% mortality were observed at the highest concentrations of spinosad, thiamethoxam, imidacloprid and indoxacarb, respectively. Correlation analysis revealed a highly significant and positive correlation between mortality of *T. castaneum* and fungal infection inhibition of both fungal species on wheat grains. Mortality of *C. ferrugineus* had only a highly significant and positive correlation with the infection inhibition of *A. parasiticus* ($p<0.01$). In conclusion, the tested insecticides proved effective in controlling *C. ferrugineus* and *T. castaneum* with indirect effect on fungal infection inhibition.

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TK and HAAK designed and performed the experiments. MRK, MU and AA helped in insect collection and bioassays. TK, HAAK and WA analyzed the data and wrote the manuscript.

Key words
Ecotoxicology, Biorational pesticides, Stored pest management, Fungal infection

INTRODUCTION

Wheat (*Triticum aestivum* L.) is amongst the important cereal crops and staple food crop for the people of Pakistan. According to the economic survey of Pakistan (2018-19), the wheat crop has a contribution of 8.9% to value-added products in the agriculture sector and 1.6% to GDP. However, the postharvest losses of wheat crop are increasing due to a number of factors. Among these, storage insect pests and diseases at postharvest stages contribute the most (Phillips and Throne, 2010). There are a number of insect pests which infest stored wheat and cause significant losses. Of these, the rusty grain beetle *Cryptolestes ferrugineus* Stephens (Coleoptera: Laemophloeidae), and the red flour beetle *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) are amongst the most serious pests of stored wheat with the potential to cause >20% postharvest losses in developing countries (Khan *et al*., 2016).

Recently, it has been reported that infestation of stored insects significantly enhances contamination with mycotoxin producing fungal in stored wheat (Khan *et al*., 2016). *Aspergillus flavus* and *A. parasiticus* are among the important mycotoxin producing fungi. Mycotoxins cause a number of health issues in humans such as acute and chronic liver diseases, nephrotoxicity, genotoxicity, tumor formation and reproductive disorders (Desjardins *et al*., 2000). Insect infestation and fungal contamination in stored grains affect seed germination that eventually reduce overall production (Phillips and Throne, 2010). Moreover, fungal attack in stored grains has been assumed a source of mycotoxins' productions that ultimately hazardous for the consumer (Birck *et al*., 2006). Stored insects play a pivotal role in fungal contamination owing to their ability to carry fungal spores on their bodies and transmit to stored grains

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mainly in two ways: (1) continuous movement of stored insects in stored commodities may help them to transfer fungal spores from one place to another and (2) feeding of stored insects usually break the seed coat that may provide a window for fungal contamination (Sétamou *et al*., 1998). Therefore, control of stored insects is of the utmost importance for managing postharvest losses and possible fungal contamination in stored grains.

The use of fumigants and conventional insecticides is currently the major tool for stored insects' control; however, it has resulted in the development of insecticide resistance (Khan *et al*., 2019). Moreover, the use of conventional insecticides and fumigants as grain protectants is questioned for their possible negative effects on the environment and human health (Vayias *et al*., 2010). Therefore, the current scenario demands the exploration of biorationals or reduced risk insecticides which effectively control stored insects and are relatively safe for the environment.

In the present study, we evaluated toxicity of selected biorational insecticides against Pakistani strains of *C. ferrugineus* and *T. castaneum*, and tested whether insects' mortality could control fungal contamination or not?

MATERIALS AND METHODS

Commodities, formulations and fungal cultures

Wheat grains of var. Seher-06 were used for bioassays. Grains were clean, free from any infestation, and had 11.1% moisture contents. Commercial formulations of four insecticides: indoxacarb (Steward 15 EC, DuPont), imidacloprid (Confidor 20 SC, Bayer CropScience), Spinosad (Tracer 24 SC, Dow Agrosciences) and thiamethoxam (Actara 25 WG, Syngenta) were used for toxicity evaluations. Fungal culture of *A. flavus* and *A. parasiticus* were acquired from the Fungus Culture Bank, Pakistan (FCBP).

Insects rearing

Adult beetles of *C. ferrugineus* and *T. castaneum* were acquired from a local stored grain market situated in Lahore (31.5497° N, 74.3436° E). Collected beetles were transported to the Entomology Lab., and reared on whole wheat grains to get pure and homogenous cultures. Beetles were reared at 26 ºC and 70±5% relative humidity.

Grain treatment and bioassays

Grain treatments and toxicity evaluation of insecticides against *C. ferrugineus* and *T. castaneum* beetles were performed following the methodology of Khan *et al*. (2016). In short, insecticide solutions of different strengths were prepared in distilled water. Each replicate was consisted of 200 g of wheat grains in a 0.5-L glass jar, and treated with different treatment combinations (Table I). Two mL solution of a specific insecticide was prepared in distilled water and applied to grains of each replicate. The grains were shaken manually for uniform distribution of the applied solution, and left for one day in complete darkness in order to evaporate solvent (distilled water). These jars were then treated with 0.5 mL spore suspension of *A. flavus* (arena "A") or *A. parasiticus* (arena "B") (10^4 spores/mL) on the upper layer of grains. After completing the grain treatments, 20 adult beetles (2-3 week old) of either *C. ferrugineus* or *T. castaneum* from the laboratory cultures were introduced into each jar. All the jars were covered with muslin cloth and kept for 14 days under complete darkness at 26ºC. All the treatment combinations were replicated five times.

Table I. Treatment combinations used in bioassays

Insecticide	Treatment	Combination
Spinosad	1	0.25 ppm+ *C. ferrugineus* or *T. castaneum*+spores
	2	0.5 ppm+ *C. ferrugineus* or *T. castaneum*+spores
	3	1 ppm+ *C. ferrugineus* or *T. castaneum*+spores
	4	*C. ferrugineus* or *T. castaneum*+spores (control)
Imidacloprid, Thiamethoxam or Indoxacarb	1	1 ppm+ *C. ferrugineus* or *T. castaneum*+spores
	2	2 ppm+ *C. ferrugineus* or *T. castaneum*+spores
	3	4 ppm+ *C. ferrugineus* or *T. castaneum*+spores
	4	*C. ferrugineus* or *T. castaneum*+spores (control)

Fungal infection inhibition percentage was observed following the protocol of Krishnamurthy *et al*. (2008). For this purpose, 60 wheat grains were taken from each treatment combination separately, and spread equally on three layers (20 per layer) of moist blotter paper discs (90 mm diameter) in glass petri plates. After seven days of incubation at 26ºC, the grains were observed using a stereo-binocular microscope. The infection inhibition was calculated as follows:

Percent infection inhibition= (number of healthy (uninfected) seeds/total number of seeds) * 100

Data analysis

The individual effect of each insecticide on beetles' mortality and subsequent fungal infection inhibition were analyzed by the one-way analysis of variance using the software Statistix 8.1. Correlation analysis was also performed to see association between insects' mortality and fungal infection inhibition.

RESULTS

Insects mortality

Spinosad

Cryptolestes ferrugineus showed complete mortality (100%) at the highest concentration of spinosad (1 ppm) in both the arenas (p<0.01; Fig. 1A-B).

Tribolium castaneum proved to be less susceptible to spinosad compared to *C. ferrugineus*. It showed 51% and 50% mortality in the arena A and B, respectively (p<0.01) at the highest concentration of spinosad. Whereas, less than 10% mortality was observed at the lowest concentration level (0.25 ppm) in both the arenas (Fig. 1C-D).

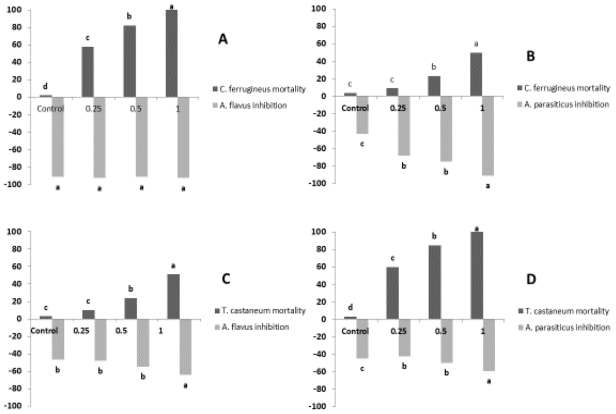

Fig. 1. Beetles' mortality (%) and subsequent fungal infection inhibition (%) at different concentrations of spinosad: (A) *C. ferrugineus* vs *A. flavus*, (B) *C. ferrugineus* vs *A. parasiticus*, (C) *T. castaneum* vs *A. flavus*, (D) *T. castaneum* vs *A. parasiticus.* In all cases, *p*<0.01.

Thiamethoxam

Cryptolestes ferrugineus showed complete mortality at the highest concentration of thiamethoxam tested (4 ppm) in both the arena (p<0.01). Almost 80% mortality was observed in the arena A and B at 2 ppm and 1ppm concentrations, respectively (Fig. 2A-B). *Tribolium castaneum* showed 96% and 98% mortality at the highest concentration of thiamethoxam (4 ppm) in the arena A and B, respectively (p<0.01). Whereas, 85 and 88% mortality were observed at 2 ppm and 1 ppm, respectively, in both the arenas (Fig. 2C-D).

Imidacloprid

The response of *C. ferrugineus* against imidacloprid was concentration dependent. It showed 93% and 97% mortality at the highest concentration of imidacloprid tested (4 ppm) in the arena A and B, respectively, followed by mortality at 2 ppm and 1 ppm (p<0.01) (Fig. 3A-B).

The response of *T. castaneum* against imidacloprid was also concentration dependent and it showed 49% and 54% mortality at the highest concentration in the arena A and B, respectively, followed by 2 ppm and 1 ppm (p<0.01) (Fig. 3C-D).

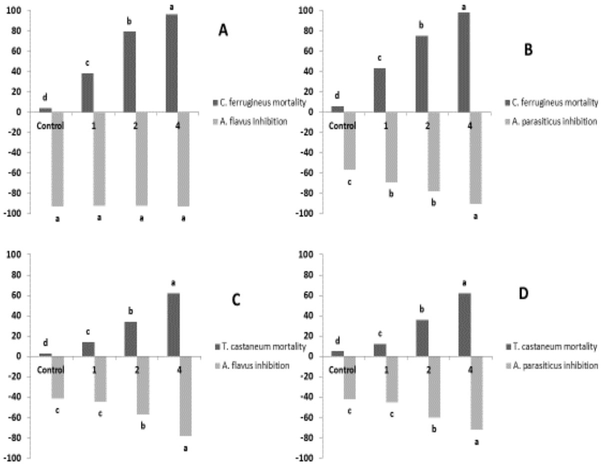

Fig. 2. Beetles' mortality and subsequent fungal infection inhibition at different concentrations of thiamethoxam: (A) *C. ferrugineus* vs *A. flavus*, (B) *C. ferrugineus* vs *A. parasiticus*, (C) *T. castaneum* vs *A. flavus*, (D) *T. castaneum* vs *A. parasiticus.* In all cases, *p*<0.01.

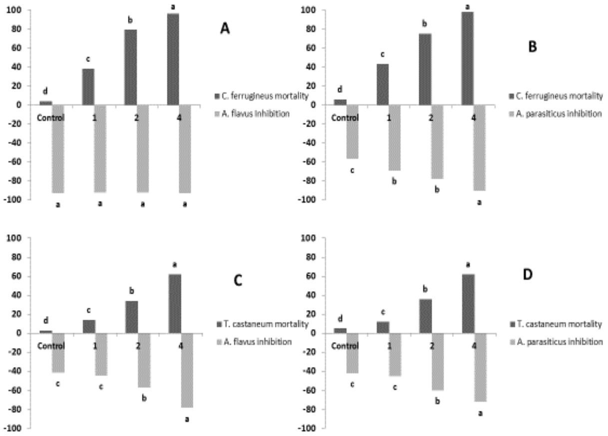

Fig. 3. Beetles' mortality and subsequent fungal infection inhibition at different concentrations of imidacloprid: (A) *C. ferrugineus* vs *A. flavus*, (B) *C. ferrugineus* vs *A. parasiticus*, (C) *T. castaneum* vs *A. flavus*, (D) *T. castaneum* vs *A. parasiticus.* In all cases, *p*<0.01.

Indoxacarb

Cryptolestes ferrugineus showed 96% and 98%

mortality at the highest concentration of indoxacarb tested (4 ppm) in the arena A and B, respectively (p<0.01). At the concentrations 2 ppm and 1 ppm, 75 and 43% mortalities were observed, respectively (Fig. 4A-B).

Tribolium castaneum showed 62% mortality at the highest concentration of indoxacarb in both arenas (p<0.01) (Fig. 4C-D).

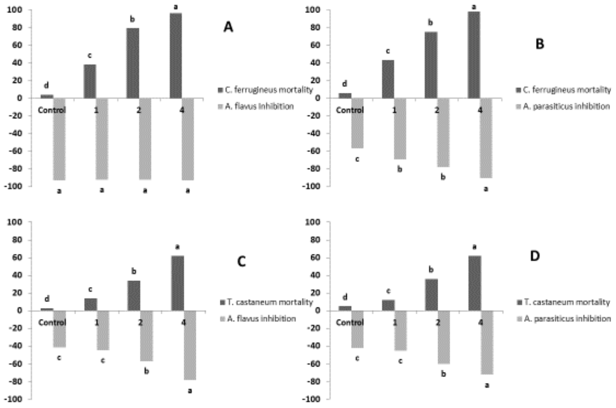

Fig. 4. Beetles' mortality and subsequent fungal infection inhibition at different concentrations of indoxacarb: (A) *C. ferrugineus* vs *A. flavus*, (B) *C. ferrugineus* vs *A. parasiticus*, (C) *T. castaneum* vs *A. flavus*, (D) *T. castaneum* vs *A. parasiticus.* In all cases, *p*<0.01.

Fungal infection inhibition

Overall, the incidence of *A. flavus* showed no association with the mortality or survival rate of *C. ferrugineus*. Despite a considerable survival rate in control and at different concentrations of tested insecticides, infection inhibition was more than 90%, which suggests that the fungal infection was independent from the activities of *C. ferrugineus*. However, infection inhibition of *A. parasiticus* was correlated with the survival rate of *C. ferrugineus*. In the case of spinosad, minimum infection inhibition (57%) was observed in the control where the survival percentage of *C. ferrugineus* was 97% and so on (Fig. 1A-B). In the case of thiamethoxam, minimum infection inhibition (47%) was observed in control where the survival percentage of *C. ferrugineus* was 94% and so on, suggesting that movement activities of *C. ferrugineus* are responsible for the incidence of *A. parasiticus* (Fig. 1C-D). In the case of imidacloprid, minimum infection inhibition (48.33%) was observed in the control where survival percentage of *C. ferrugineus* was 95% and so on, suggesting that movement activities of *C. ferrugineus* are responsible for the incidence of *A. parasiticus* in treated wheat samples (Fig. 3A-B). In the case of indoxacarb, minimum infection inhibition (56.67%) was observed in the control where survival percentage of *C. ferrugineus* was 94% and so on (Fig. 4A-B).

Fungal infection by both of the fungal species seems to be associated with the survival rate of *T. castaneum*, suggesting that movement of the surviving insects most probably aided in the incidence of both fungal species. In the case of spinosad, maximum inhibition (64.33%) of *A. flavus* was observed at 1 ppm, where 51% mortality of *T. castaneum* was observed. Whereas, minimum inhibition (46%) was observed in control versus 3% mortality of *T. castaneum*. Similarly, in the case of *A. parasiticus*, maximum inhibition (59%) was observed at the highest concentration where 50% mortality of *T. castaneum* occurred (Fig. 1C-D). In the case of thiamethoxam, maximum inhibition (92%) of *A. flavus* was observed at 4 ppm, where 96% mortality of *T. castaneum* has been observed. Whereas, minimum inhibition (40%) was observed in control versus 2% mortality of *T. castaneum*. Similarly, in the case of *A. parasiticus*, maximum inhibition (93.33%) was observed at the highest concentration where 98% mortality of *T. castaneum* occurred (Fig. 2C-D). In the case of imidacloprid, maximum inhibition (73%) of *A. flavus* was observed at 4ppm, where 49% mortality of *T. castaneum* has been observed. Whereas, minimum inhibition (40-44%) was observed at rest of the concentrations and control (all were statistically similar). Similarly, in the case of *A. parasiticus*, maximum inhibition (73%) was observed at the highest concentration where 54% mortality of *T. castaneum* occurred (Fig. 3C-D). While in the case of indoxacarb, maximum inhibition of *A. flavus* was observed at 1ppm indoxacarb and in control, where survival rate was 86 and 97%, respectively. Similarly, in the case of *A. parasiticus*, maximum inhibition (72%) was observed at the highest concentration where 62% mortality of *T. castaneum* occurred (Fig. 4C-D).

Correlation between insects' mortality and fungal infection inhibition

Correlation analysis revealed a highly significant and positive correlation between mortality of *T. castaneum* and fungal infection inhibition of both species. Mortality of *C. ferrugineus* had only a highly significant and positive correlation with the infection inhibition of *A. parasiticus* (p<0.01; Table II).

Table II. Correlation analysis between insects' mortality and fungal infection inhibition.

	Aspergillus flavus	***Aspergillus parasiticus***
Tribolium castaneum	0.92**	0.92**
Cryptolestes ferrugineus	0.06 ns	0.95**

DISCUSSION

The present study revealed varying toxicity of selected biorational insecticides against *C. ferrugineus* and *T. castaneum*, and tested insecticides had indirect influence on fungal infection inhibition. Spinosad proved to be the most toxic insecticide against *C. ferrugineus* compared to *T. castaneum*. *Cryptolestes ferrugineus* showed complete mortality at the highest concentration of Spinosad, while *T. castaneum* exhibited about 50% mortality. Spinosad is a microbial insecticide and has been assumed eco-friendly with low toxicity towards human beings. For this reason, it is used against a number of agricultural pests, including insect pests of stored commodities. Previous studies revealed toxic potential of spinosad against different stored insects. For instance, spinosad caused complete mortality of *R. dominica* even at 0.25 ppm, and 94% mortality of *S. oryzae* at 1 ppm (Khan *et al*., 2016). In a previous report, spinosad caused complete mortality of *R. dominica* beetles at 1 ppm, but least effective against *T. castaneum* (Nayak *et al*., 2005).

Cryptolestes ferrugineus showed complete mortality at the highest concentration of thiamethoxam tested (4 ppm), whereas, *T. castaneum* showed 96% and 98% mortality at the highest concentration of thiamethoxam (4 ppm) in the arena A and B, respectively. Thiamethoxam is a neonicotinoid and first evaluated against insect pests of stored commodities by Arthur *et al*. (2004) and found effective in controlling *R. dominica* and *S. oryzae* under laboratory conditions, but least effective against *T. castaneum*. In a recent report from Pakistan, *S. oryzae* exhibited <100% mortality at 2 ppm after 14 days of exposure (Khan *et al*., 2016). The difference in toxicities was assumed to be due to different origins of the insect species.

In the present study, mortality of *C. ferrugineus* and *T. castaneum* were dependent on imidacloprid concentrations. *Cryptolestes ferrugineus* showed 93% and 97% mortality at the highest concentration of imidacloprid tested (4 ppm) in the arena A and B, respectively. *Tribolium castaneum* exhibited 49% and 54% mortality at the highest concentration in the arena A and B, respectively, and there was a more gradual decrease in mortality as the concentration of imidacloprid decreased. Imidacloprid is also a neonicotinoid and has been tested against different stored insect pests. In a recent report, *R. dominica* exhibited 94% mortality when exposed to 4 ppm concentration of imidacloprid as compared to 39% mortality of *S. oryzae* at the same concentration (Khan *et al*., 2016).

In the present study, indoxacarb proved to be most toxic against *C. ferrugineus* compared to *T. castaneum*. *Cryptolestes ferrugineus* showed 96% and 98% mortality at the highest concentration of indoxacarb tested (4 ppm) in the arena A and B, respectively. *Tribolium castaneum* revealed 62% mortality at the highest concentration of indoxacarb in both arenas. Recently, toxicity of indoxacarb has been evaluated against *R. dominica* and *S. oryzae* and *T. confusum* (Miliordos *et al*., 2017). The results revealed that indoxacarb was effective in controlling *R. dominica* and *S. oryzae*, but not for *T. confusum*. Similarly, Khan *et al*. (2016) reported that indoxacarb was more toxic to *R. dominica* than *S. oryzae*.

With respect to fungal infection inhibition, tested insecticides had an indirect effect of insecticides on fungal infection inhibition of the tested fungi. It was observed that mortality of *T. castaneum* when exposed to any insecticides resulted in the reduced fungal infection of both fungi. Mortality of *C. ferrugineus* had positive correlation with infection inhibition of *A. parasiticus* only. From these data, it can be assumed that the tested insecticides reduced the movement of insects in grains by causing mortality. As a result of insects' mortality, feeding activity by damaging the seed coat of grains would also be reduced, which ultimately lead to inhibit fungal infection in grains (Khan *et al*., 2016). When insect pests attack stored commodities, they move from one place to another for feeding and other life activities. During this, they have the ability to transfer fungal spores from infected to healthy grains (Nesci *et al*., 2011). Moreover, stored insect pests damage seed coat of grains as a result of feeding that ultimately provide an entry point for fungal infections. Therefore, the data of the current study revealed severe in bioassays where the survival rate of insects was high, except in the case of *C. ferrugineus* versus *A. flavus*. This might be due to the inability of *C. ferrugineus* to transfer *A. flavus* spores since it has been reported that the spread of fungal spores is highly dependent on insect species involved (Lamboni and Hell, 2009).

CONCLUSION

In conclusion, the tested insecticides proved effective in controlling *C. ferrugineus* and *T. castaneum* with indirect effect on fungal infection inhibition. Further studies are needed under field conditions to check the effectiveness of these insecticides and ultimate effect on fungal infection inhibition.

Statement of conflict of interest

The authors have declared no conflict of interest.

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