Research Article

Enhanced Activities of Metabolic Enzymes Associated with Insecticide Resistance in Red Flour Beetle (*Tribolium castaneum* Herbst)

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Abstract | The mechanism of resistance was determined in four different field populations of red flour beetle (*Tribolium castaneum*) collected from Faisalabad (Fsd-strain), Jhang (Jh-strain), Sahiwal (Swl-strain) and Sargodha (Sgd-strain) districts of Pakistan. Bioassays with two frequently used insecticides, *i.e.* deltamethrin and permethrin and one biological insecticide, spinosad, were performed on these strains of *T. castaneum*. Toxicological studies demonstrated that Fsd-, Jh- and Swl- strains exhibited higher tolerance ratio of 26.5-fold, 21-fold and 18.6-fold respectively relative to Sgd-strain against deltamethrin, whereas, these strains did not have significantly higher tolerance ratio against permethrin and spinosad except Fsd-strain with 9.5-fold tolerance ratio against permethrin. Biochemical studies indicated that Swl-strains had significantly higher activities of cellulases. Enzyme kinetics results demonstrated that Swl-strain had a maximum value of Vmax (68.4 Nm s^{-1}) and lowest value of Km (12.8 g L^{-1}). Fsd- and Swl-strains exhibited increased activities of amylases with Vmax value of 2.68 and 1.89 µM s^{-1} respectively and Km value of 23.8 g L^{-1} and 40.1 g L^{-1}, respectively. These findings suggest that the increased activities of cellulases and amylases may play a major role in mitigating fitness-cost associated with insecticide resistance.

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Introduction

Stored grain sector has been vulnerable to many kinds of insect pest infestations throughout the world. In Pakistan, these insect pests can cause considerable damage reaching up to 10-20% (Ahmed, 1983; Khanzada et al., 2011). Among stored grain insect pests, red flour beetle (*Tribolium castaneum* Herbst; Coleoptera: Tenebrionidae) is the major pest in different anthropogenic structures used for the processing and storage of grain-based products (e.g., flour mills, warehouses, retail stores). This species infests a wide range of commodities including grains, flour, peas, beans, cacao, dried fruits, spices, cereals, pasta, dried pet food, chocolate, nuts and seeds but milled grain products such as flour appear to be the preferred food of *T. castaneum* (Good, 1936; Weston and Rattlingourd, 2000). Its infestation in stored foods directly affects both the quantity and quality of the commodity (Mondal, 1994).

At present, insecticides belonging to different groups of pesticides such as fumigants (phosphine and methyl bromide), organochlorides, organophosphates,

pyrethroids and insect growth regulators, are being used for the control of *T. castaneum*. Due to successive and irrational use of the insecticides, this species has become resistant to these insecticides (Zettler and Cuperusi, 1990; Jagadeesan et al., 2012; Kocak et al., 2015). Resistance to pesticides among native populations of *T. castaneum* is widespread throughout the world including Pakistan (Andreev et al., 1999; Awan et al., 2012). Increased activities of catalases and amylases are also associated with resistance in *T. castaneum* (Awan et al., 2012). Similarly, increased serine- and cysteine-proteolytic and cellulolytic activity, and kinetic parameters in insecticide-resistant strains suggest that these enzymes are more important in mitigating the cost of insecticide resistance in maize weevil strains (Araujo et al., 2008).

As insect pests, including *T. castaneum*, have been found developing resistance and/or tolerance to certain chemical insecticides (Collins, 1990), a constant monitoring of this phenomenon in local pest populations is required in order to rationalize the insecticide use against *T. castaneum*. Furthermore, new biorational strategies such as use of bio-pesticides in stored grain pest management, as alternate to conventional synthetic insecticides, could be a safer and environment-friendly option. In this regard, spinosad, a novel neuro-toxicant derived from soil actinomycete, *Sacharopolyspora spinosa* (Thompson et al., 2000), is considered as a potential grain protectant for stored grain pest management (Hertlein et al., 2011). It exhibits less environmental risk and less toxicity to human-being as compared to chemical insecticides. Its efficacy has already been evaluated on wheat against adults of *T. castaneum* and other stored pests and was found effective against all these pests (Fang et al., 2002; Flinn et al., 2004).

The present research work was aimed at monitoring the level of resistance in field population, screening different populations of *T. castaneum* collected from four divisions (*i.e.* Faisalabad, Jhang, Sahiwal and Sargodha) of Punjab (Pakistan) for resistance against two pyrethroids (deltamethrin, permethrin) and a bio-pesticide (spinosad), and to determine the mechanism of metabolic resistance manifested by these strains against these insecticides. Two metabolic enzymes, cellulases and amylases, were measured in different populations of *T. castaneum*.

Materials and Methods

This study was carried out on the *T. castaneum* that was reared in the laboratory under controlled conditions (25±2 ˚C and 55±2% RH) in plastic jars containing sterile breeding media such as containing a mixture of whole wheat flour (0.5 kg), dried yeast (1 g). Insects of uniform age and size are used for the experiment. The jars were closed by muslin cloth. Four strains of *T. castaneum* collected from market and storage structures of districts Sargodha, Jhang, Faisalabad and Sahiwal. The population from Sargodha was reared in laboratory with insecticidal pressure for twenty generations and was used as susceptible population in the study.

Bioassays were performed on *T. castaneum* strains with deltamethrin, permethrin, and spinosad. Five concentrations of each insecticide ranging mortality from 0 to 100 percent with three replicates were used. Twenty beetles of homogeneous size and age were released on 9cm diameter insecticide treated filter paper in glass Petri dishes. In order to avoid the mortality due to starvation, 1 g sterilised broken wheat grains dipped in same insecticide concentration were also added to the replicates. Mortality was counted after 24h intervals.

The mortality data was analysed by probit analysis (Finney, 1971) with POLO-PC software to determine the median lethal concentration (LC_{50}), 95% confidence intervals (CIs). The mortality was corrected by using Abbott's formula (Abbott, 1925) if it occurred in control. The tolerance ratio (TR_{50}) was determined as the LC_{50} of resistant population divided by the LC_{50} of the susceptible population. LC_{50} values of resistant populations were considered as non-significantly different ($P > 0.05$) when their CIs overlapped (Litchfield and Wilcoxon, 1949).

Activities of cellulases and amylases were measured in different strains of *T. castaneum*. For enzyme analysis crude fraction prepared by homogenizing 100 g of stored beetle with pestle and mortar using 400 mL 0.2 M Sodium phosphate buffer of pH 6.6 and this mixture was stored overnight in cool incubator. 10 mL of 2 mM EDTA, Triton X-100 was added in the sample and homogenized with the help of homogenizer. The homogenized sample was centrifuge at 6000 rpm for 15 min to separate the supernatant and filtered for further processing. About 100 mL of fil-

tered supernatant was mixed with 400 mL of chilled acetone (1:4 of methanol) and kept the mixture overnight at low temperature of 4°C to precipitate the proteins. During the protein precipitation, the chilled acetone helps to avoid denaturing of enzymes. It was centrifuged again at 6000 rpm for 15 min to get a pallet. The pallet was air dried and dissolved in 0.2 M sodium-phosphate buffer of pH 6.6 and was used for further analysis. This solution was stored at 4°C.

Bradford assay (Bradford, 1976) was used for the estimation of total protein in sample. Standard curve of bovine serum albumin was prepared. A quantity of Bradford reagent was added to 100 µL of protein sample and absorbance was measured at 595 nm against reagent blank after 5 min. Protein concentration was calculated by plotting a standard curve.

Enzyme assay were performed using Han and Srinivasan (1969) modified 2, 2-dinitrosalicylic acid method to measure the amount of reducing sugar by taking the glucose as standard. For the DNS reagent preparation, 10 g of 2, 2-dinitrosalicylic acid was dissolved in 200 mL 2 M Sodium Hydroxide (NaOH) followed by preparation of 500 mL 60% Sodium Potassium tartrate. The solutions were mixed together to make the final volume up to 1000 mL and filtered them. For the reducing sugars, preparation of reference blank was done by taking 50 µL enzyme solution of the same dilution, 500 µL of 2.5% CMC for Cellulase, 3% starch for amylase and heated in a boiling water bath for 15 min. Standard glucose curve was made for measuring concentration of released glucose, enzyme activity was determined in UmL^{-1}.

Cellulase activity (CMC Assay) assay was performed by the preparation of reaction mixture by adding 50 µL of crude enzyme sample to 500 µL of CMC solution (2.5% w/v) and 500 µL of 0.2 M sodium phosphate buffer (pH 6.6). Then the incubation of this mixture was done overnight at 50 °C with gentle shaking. After 24 hr of incubation, 3 mL of DNS reagent was added to enzyme mixture and incubation had done in the boiling water for 15 min after that filtered the reaction mixture. Absorbance of the sample was measured at 540 nm against a reagent blank that had prepared by adding 50 µL distilled water, 500 µL of sodium phosphate buffer (pH 6.6) and 500 µL of 2.5% CMC. Enzyme kinetics was measured for different strains by using the different concentration of substrates (10-40 gL^{-1} CMC). Enzymes activities in different strains were compared by ANOVA and means were compared by DMR test (Duncan Multiple Range Test).

Amylase activity (starch assay) assay was performed by the preparation of reaction mixture by adding 50 µL of crude enzyme sample to 500 µL of starch solution (3% w/v) and 500 µL of 0.2 M sodium phosphate buffer (pH 6.6). Then the incubation of this mixture was done overnight at 60°C with gentle shaking. After 24 hr of incubation 3 mL of DNS reagent was added to enzyme mixture and incubation was done in the boiling water for 15 min after that filtered the reaction mixture. Absorbance of the sample was measured at 540 nm against a reagent blank that was prepared by adding 50 µL distilled water, 500 µL of sodium phosphate buffer (pH 6.6) and 500 µL of 3% starch. Enzyme kinetics was measured for different strains by using the different concentration of substrates (20-50 g L^{-1} starch). Inter-strains comparison for enzyme activity was performed as described above.

Results and Discussion

The bioassay results of four strains with deltamethrin indicated that Fsd-strain has LC_{50} value of 130.3. This strain has highest value of LC_{50} among the tested strains while, Sgd-strain was found least tolerant to deltamethrin. So, tolerance ratio was calculated in accordance to the Sgd-strain. Tolerance ratio 50 (TR_{50}) of Fsd-strain was 26.5 and it was significantly different from that of Sgd-strain. LC_{50} of Jh-, Swl- and Sgd-strain have LC_{50} values 109.3, 91.4 and 4.9 ppm with TR50 values 21, 18.6 and 1, respectively (Table 1). Tolerance of Jh- and Swl-strain was significantly different from Sgd-strain. The permethrin toxicity data of four strains showed that Fsd-strain had highest value of LC_{50} among the tested strains. Jh-strain was found least resistant to permethrin. So, tolerance ratio was calculated with respect to the Jh-strain. Fsd-strain has LC_{50} value of 101.1 ppm with a TR_{50} of 9.59, which was significantly different from Jh-strain. Swl- and Sgd-strains have LC_{50} values of 13.6 and 11.4 ppm with TR50 values of 1.29 and 1.09, respectively, which were not significantly different from Jh-strain (Table 1). The data of spinosad toxicity showed that Sgd-strain has highest value of LC_{50} among the tested strain. Fsd-strain was found least tolerant to spinosad. Therefore, TR_{50} was determined in accordance to the Fsd-strain. Sgd-strain had LC_{50} value of 64.9 ppm with a TR_{50} value of 3.63,

which was not significantly different from Fsd-strain. Similarly, Jh- and Swl-strains have LC_{50} values of 63.5 and 61.5 ppm with TR_{50} values of 3.55 and 3.44 respectively, which are not significantly different from Fsd-strain (Table 1).

Table 1: *Tolerance level of different strains of T. castaneum adults to insecticides.*

Name of Strain	LC_{50} (ppm)	CI (95%)	Fold increase tolerance (TR_{50})Φ	Significance
deltamethrin				
Faisalabad	130.3	117.5-144.4	26.5	*
Jhang	109.3	96.3-123.2	21	*
Sahiwal	91.4	77.4-105.6	18.6	*
Sargodha	31.4	25.6-36.5	6.4	*
Susceptible	4.9	3.6-6.5	1	--
permethrin				
Faisalabad	101.10	90.1-112.6	9.59	*
Jhang	13.69	12-15.5	1.29	NS
Sahiwal	11.47	10-13	1.09	NS
Sargodha	11.9	9.7-13.4	1.12	NS
Susceptible	10.54	9.1-12.1	1	--
spinosad				
Faisalabad	64.97	57.7-74.9	3.63	*
Jhang	63.51	55.8-72	3.55	*
Sahiwal	61.55	52-72.1	3.44	*
Sargodha	40.4	34.9-44.2	2.26	*
Susceptible	17.87	13-21	1	--

Φ TR_{50} (Tolerance ratio 50 = lethal concentration 50 of tested strains / lethal concentration of susceptible-strain).

Analysis of variance has been performed on the cellulases activities. ANOVA table showed that the activities of cellulases were significantly different. Means of cellulases activities were compared by using Duncan's Multiple Range Test (DMR). Data illustrated that maximum cellulase enzyme activities were found in Swl-strain with mean value of 2.03 U mL^{-1} and it was significantly different from those of Sgd-, Fsd- and Jh-strains, having mean cellulases activities of 1.57, 1.55 and 1.46 U mL^{-1}, respectively without any significant difference. Jh-strain showed the lowest cellulase activities (Figure 1). The Michaelis-Menten plot (Figure 2) was used to determine the Vmax (maximum velocity) and Km (Michaelis constant) values of different strains. The data showed that Swl-strain had maximum value of Vmax (68.4 Nm s^{-1}) which indicates the higher conversion rate of substrate to products and enzyme efficiency. Fsd-, Sgd- and Jh-strains have Vmax values of 57.5, 51.4 and 50.5 Nm s-1, respectively. Km value indicates the affinity of enzyme with the substrate. Swl-strain had a Km value of 12.8 g L^{-1}, which is a minimum value as compared to other strains, thus this strain has maximum affinity with the substrate. While other strains Fsd-, Sgd- and Jh-strain have less affinity with the substrate and have Km values of 19.8, 18.6 and 24.2 g L^{-1}, respectively.

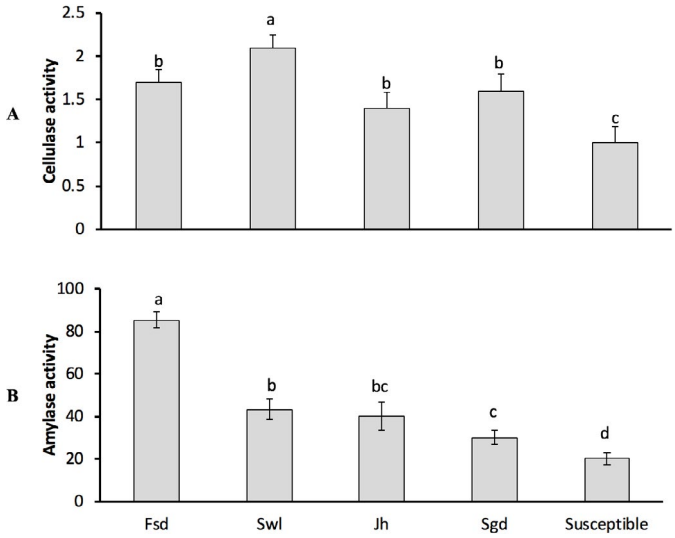

Figure 1: *Cellulase and amylases activities in different strains of T. castaneum adults.*

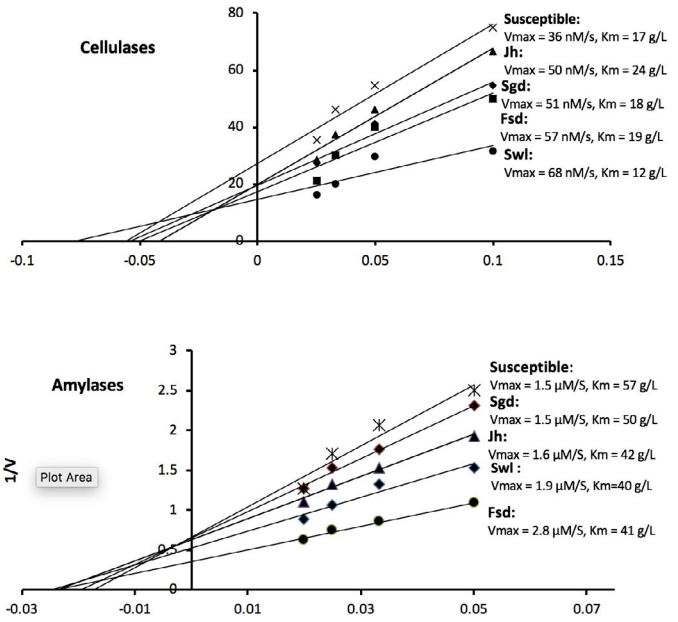

Figure 2: *Line-weaver Burk plots for determining the kinetic constants (Vmax and Km) of cellulases and amylases in different strains of T. castaneum adult. Crude enzyme extract (50 µL) was incubated with 10-40 and 20-50 g L^{-1} of starch for cellulases and amylases respectively.*

Analysis of variance showed that the activities of amylases were significantly different among the strains. Data analysis showed that Fsd-strain exhibited maximum amylase activities with a mean value of 85 UmL^{-1} and it was significantly different from Sgd-, Swl- and

Jh-strains. Mean values of Swl- and Jh-strains were 44.3 and 39 U mL^{-1}, respectively and were insignificantly different from each other, whereas, amylase activities of Swl-strain were significantly different from Sgd-strain (mean value of 34 UmL^{-1}). Sgd-strain was having the lowest activities (Figure 1). The Michaelis-Menton plot data described that Fsd-strain had a Vmax value of 2.68 µM s^{-1}, showing a higher conversion rate of substrate to products and enzyme efficiency. Swl-, Jh- and Sgd -strains had Vmax values of 1.89, 1.58 and 1.52 µM s^{-1}, respectively. Fsd-strain had a Km value of 23.8 g L^{-1}, which was a minimum value as compared to other strains, thus this strain had a maximum affinity with the substrate. While other strains Swl-, Jh-, and Sgd-strains have comparatively less affinity with the substrate and have higher Km values (40.1, 41.9 and 50.6 g L^{-1}, respectively).

Red flour beetle (*T. castaneum*) is one of the major stored grain insect pests. *T. castaneum* has been reported to develop resistance against many of the synthetic chemical insecticides (Rahman and Shahjahan, 2000; Baki et al., 2005). The present study was conducted to monitor the phenomenon of resistance in *T. castaneum*. In order to monitor the mechanism of resistance in *T. castaneum*, samples of *T. castaneum* infested grains were collected from the markets and storage structures of Faisalabad, Jhang, Sahiwal and Sargodha Divisions of Punjab (Pakistan). Three insecticides, deltamethrin, permethrin and spinosad, were used for bioassay to evaluate the level of resistance or tolerance in different populations of *T. castaneum*.

Tolerance of insecticides in native populations of T. castaneum

We could not get the susceptible strain of *T. castaneum*. Therefore, tolerance was assessed relative to most susceptible strains among the strains used for bioassays. The tolerance determined is not absolute; it is relative to the most susceptible one. The activities of metabolic enzymes (cellulases and amylases) were determined in four strains of this beetle.

In Pakistan, deltamethrin is widely used both in public and private sectors to control *T. castaneum* and other insect pests, either as pre-storage pest eradication in structures or used directly on stored commodity such as bagged grains, cereals etc. Our results explicate that Fsd-strain has higher tolerance ratio to deltamethrin (26.5-fold) and to permethrin (9.59-fold) (Table 1). Jh- and Swl-strains also have higher tolerance ratio of 21 and 18.6, respectively, to deltamethrin. However, it is surprising that Sgd-strain has not acquired as much tolerance to deltamethrin, permethrin and spinosad (Table 1). Cluster analysis also revealed that Sgd-strain exhibits least tolerance to deltamethrin and is present at large distance from Fsd-, Swl- and Jh-strains (Figure 1) in tolerance. In contrast, Fsd-strain showed slightly higher tolerance to permethrin and is also present at large distance from Sgd, Swl- and Jh-strains in tolerance (Figure 2). Although, Fsd-strain is separated from other strains in tolerance, yet all four strains are susceptible to spinosad (Figure 2). Low level of tolerance in Sgd-strain could be due to less frequent use of this insecticide on the storage structures and bags in Sargodha. Similarly, increased tolerance in Fsd-strain could be due to the excessive use of deltamethrin on storage structures. Deltamethrin resistance is primarily associated with detoxification by enhanced activities of metabolic enzymes such as catalase, amylase, cellulases, cytochrome P450s and acetyl-cholinesterase (Araujo et al., 2008; Awan et al., 2012; Chandor-Proust et al., 2013; Hussain et al., 2015).

Evolution of resistance in the *T. castaneum* against several insecticidal threatens sound storage products. More alarming information is that the high resistance to deltamethrin resulted in cross resistance (380 folds) against spinosad, whereas, field strains were susceptible to spinosad (Awan et al., 2012). It is concluded that the resistance to deltamethrin in *T. castaneum* is not due to a single mechanism; rather, resistance is combined multi-mechanism.

On the basis of our results, it is recommended to stop the usage of deltamethrin in those regions where resistant strains are present such as in Faisalabad. It is clear from our results (Table 1) that the tested strains were susceptible to permethrin and spinosad except the Fsd-strain (TR50 = 9.5 fold) which was found slightly tolerant to permethrin. Thus, insecticides, permethrin and spinosad, could be used as alternative to deltamethrin. While between the permethrin and spinosad, spinosad is a reduced-risk pesticide. Spinosad has been found effective against adults of the stored grains pests (*Rhyzopertha dominica* (F.); *O. surinamensis* (L.); *Sitophilus oryzae* (L.); *Plodia interpunctella* (Hübner) and *T. castaneum* (Herbst)) and was considered as a potential grain protectant for stored grain management (Fang et al., 2002). Spinosad treatment of wheat is usually suggested as an effective control

strategy in suppressing the stored wheat pest (Flinn et al., 2004). Hence, it could be used as an alternative insecticide against stored grain pest.

Activities of metabolic enzymes and tolerance to insecticides

We found that Swl-strain has significantly higher activities of cellulases as compared to all other strains (Figure 1). This strain also has a maximum value of Vmax (68.4 Nm s-1) and lowest value of Km (12.8g L^{-1}) (Figure 2). These two parameters (Vmax and Km) explain the high reactivity of this enzyme with higher affinity for substrate. However, other strains have less value of Vmax such as 57.5, 51.4 and 50.5 Nm s^{-1} for Fsd-, Sgd and Jh-strains, respectively. It is noteworthy that Swl-strain showed higher resistance to deltamethrin (18.6-fold). Probably it is due to the higher activities of cellulases found. Results of the present study regarding amylases activities present that Fsd-strain has significantly higher activities of amylases 85 (U mL^{-1}) (Figure 1) in the adults of *T. castaneum*. As Fsd-strain exhibits higher tolerance ratio to deltamethrin (26.5-fold) and permethrin (9.59-fold) as shown in Table 1. Similarly, Jh- and Swl-strains also have higher tolerance ratio of 21- and 18.6-folds, respectively to deltamethrin. It is probable that tolerance in three strains (Fsd-, Jh- and Swl-strain) of *T. castaneum* is due to higher activities of amylases. But only Swl-strain could have tolerance due to increased activities of cellulases and amylases. Swl-strain had increased activities of cellulases and showed non-sensitive behaviour to insecticides as compare to others, while Jh-strain showed the lowest cellulase activities (Figure 1) and more susceptibility to all insecticides studied (Table 1).

It is supposed that increased activities of cellulases also increase the tolerance of *T. castaneum* strain to deltamethrin. Insects are totally dependent on α-amylase and cellulases for their survival, these enzymes are good target candidates for bio-insecticides. Insecticide resistance may be mitigated by increased energy accumulation and mobilization. Preliminary evidence in the maize weevil (*Sitophilus zeamais,* Coleoptera: Curculionidae) suggested some possible involvement of amylases and cellulases in such phenomenon. Such results provide support for the hypothesis that enhanced α-amylase and cellulase activity may be playing a major role in mitigating fitness costs associated with insecticide resistance (Araujo et al., 2008; Lopes et al., 2010). But increased activities of enzymes in resistant strains are explained by different molecular phenomena occurring in insects and lead to metabolic resistance. Metabolic resistance is the qualitative or quantitative changes in the enzymes, which metabolise the insecticides before they reach their target sites. The gene coding for the enzymes may be amplified so that an over-production of the enzymes can occur. An over-expression of genes can also occur due to a change in the gene regulatory system (increased transcription) or to increased mRNA stability. Finally, the resistant insects may possess a mutant gene so that the enzyme has higher catalytic centre of activities toward insecticides (Hemingway and Karunaratne, 1998; Li et al., 2007; Hussain et al., 2015).

The above results need more confirmation and verification at molecular level by observing the expression of genes gearing the metabolic enzyms complex in resistance strains or the utilisation of high throughput molecular techniques (*e.g.* mRNAseq etc) and hence constitute the future perspective of this research.

Conclusions and Recommendations

Conclusively, the present study findings recommend a regular monitoring of the phenomenon of resistance and/or tolerance in different native populations of *T. castaneum*, avoiding the repeated and excessive use of synthetic and conventional insecticides and promoting the utilization of alternate novel-chemistry insecticides that exhibit less toxicity and persistency.

Author's contribution

MAR conceived the idea. JC collected insect strains and performed experiments. MAR and MZM analysed the data. MAR and JC wrote the manuscript. MA and SA provided technical assistance and supervised the experiments. SK revised the manuscript. SA and MZM performed proofreading of the final manuscript.

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