

## Toxicological and Biochemical Studies on Spinosad and Synergism with Piperonyl Butoxide in Susceptible and Resistant Strains of *Tribolium castaneum*

Daud A. Awan,<sup>1,2</sup> Mushtaq A. Saleem,<sup>1</sup> Muhammad Shahid Nadeem<sup>2</sup> and A. R. Shakoori<sup>2\*</sup>

<sup>1</sup>University College of Agriculture, Bahauddin Zakariya University, Multan, Pakistan

<sup>2</sup>School of Biological Sciences, University of the Punjab, Lahore, Pakistan

**Abstract.-** Evolution of resistance in the red flour beetle *Tribolium castaneum* against several insecticides threatens sound storage of stored grain products. Dose-response bioassays were conducted on area-wise collection of beetle populations from major wheat growing cities of Pakistan, while biochemical techniques were used to evaluate basic mechanisms underlying resistance. All the collected field strains were susceptible to spinosad. High resistance to deltamethrin resulted in cross resistance (380 folds) against spinosad just up to 8 generations. Spinosad and deltamethrin resistance is primarily associated with detoxification by enhanced levels of several enzymes. Enzymatic assays showed that catalase, amylase and acetylcholinesterase activities, but not phosphatases (alkaline and acidic), were positively correlated with resistance to deltamethrin and spinosad. Piperonyl butoxide (PBO) resulted in significant synergism with spinosad as LC<sub>50</sub> value was greatly reduced *i.e.* from 231375.2 to 305.4ppm when used in 1:4 against R-MDA strain. Knowledge of the mechanisms involved in spinosad and deltamethrin cross resistance is a key to devising new resistance management strategies aimed at restoring the efficacy of spinosad-based programmes.

**Key words:** *Tribolium castaneum*, spinosad, catalase, amylase, acetylcholinesterase, phosphatases (alkaline and acidic), piperonyl butoxide.

### INTRODUCTION

**R**ed flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is considered as one of the best known, major and serious pests of wheat flour and other grain products in Pakistan (Saleem and Shakoori, 1989) and several other countries of the world (Howe, 1965; Champ and Dyte, 1977). Annual post-harvest losses resulting from insect damages, microbial deterioration and other factors are estimated to be 10-25% of worldwide production (Mathews, 1993). To control this and many other important pest insects, our ability is seriously threatened by toxic residues of insecticides on stored grains, toxicity to consumers, increasing cost of application and widespread resistance problem, particularly in the warmer regions of the world (Dyte and Blackman, 1970; Champ and Dyte, 1977; Saleem and Shakoori, 1989). Therefore, there is an urgent need to develop safe alternatives that are of low cost, convenient to use and environmental friendly. The development of

environmental friendly insecticides, having specificity to insects along with low toxicity to vertebrates, has captured worldwide attention of scientists (Ishaaya and Degheele, 1998).

Spinosad is considered as a reduced risk insecticide based on metabolites of a soil bacterium, *Saccharopolyspora spinosad* (Mertz and Yao, 1990). This bacterial insecticide has a unique mode of action with a very low mammalian toxicity compared with other insecticides (Bert *et al.*, 1997; Thompson *et al.*, 1997, 2000). It is a stomach poison with contact action and shows some control on small beetle larvae (Thompson *et al.*, 2000; Tjosvold and Chaney, 2001). It kills insects through the activation of acetylcholine nervous system through nicotinic receptors. Within 48 h, 60-80% of spinosad or its metabolites are excreted through urine or feces, in rats (Dow, 1997; EPA, 1997). It has been used for the control of insect pests in the orders Lepidoptera, Diptera and Thysanoptera and some species of Coleoptera and Orthoptera (Thompson *et al.*, 2000). Laboratory and field data have shown spinosad at 1 mg (a.i)/kg to be effective against several major stored-grain insects (Fang *et al.*, 2002a, 2002b; Toews *et al.*, 2003; Huang and Subramanyam, 2004).

\* Corresponding author: [arshak@brain.net.pk](mailto:arshak@brain.net.pk)  
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Synergists could play an important role in combating resistant population of *T. castaneum* and other insect pests, as well as reduction of insecticidal application rates, in potential environmental contamination and improvement in the performance of integrated pest management programs. The presence of pesticides in the environment not only increases the non-target organism mortality, but also their levels can be reached where mammals ultimately can be seriously affected (Baki *et al.*, 2005). Therefore the introduction of botanical pesticides or plant extract as synergist could be greatly beneficial, both economically and ecologically, especially since tests have shown that synergism increases toxicity of insecticides against insects and not mammals (Champ and Campbell-Brown, 1970).

Considering the above mentioned threats and novelty of spinosad, the present study was carried out to determine its efficacy, synergism with PBO and biochemical basis of its effects against resistant, susceptible and field collected strains of *T. castaneum* from major wheat growing regions of Pakistan.

## MATERIALS AND METHODS

### *Biological materials*

About 30-100 adult beetles of *T. castaneum* were collected from different godowns at farmer level, government stores and flour/feed mills located in major wheat growing cities of Pakistan such as; D G Khan (DGK), Multan (MUL), Vehari (VRI), Faisalabad (FSD), Chaniot (CHT), Jhung (JNG), Lahore (LHR), Sahiwal (SWL) and Karachi (KCR). Phosphine resistant (R-Phos) strain was collected from University of Karachi while reference strain was obtained from University of Sussex, (Lab UK). Deltamethrin resistant (R-MDA) as well as deltamethrin susceptible (S-MDA) strains were developed in the laboratory. All the adults were reared in glass jars (300 mL) on wheat flour mixed with yeast (10:1 w:w) sterilized at 60°C for 90 min covering the mouth with fine cloth to restrict either escape of the adults or entry of mites. The culture was kept in a temperature controlled room maintained at 30±1°C and 65±5% relative humidity at the Stored Product Insect Laboratory, Department

of Entomology, University College of Agriculture, Bahauddin Zakariya University, Multan, Pakistan in year 2006-07, without any loss of fitness. After 72 h, the culture was sieved through 500µm mesh to separate the adults and the eggs from the flour to maintain the subculture. In this experiment, 30 larvae of 6<sup>th</sup> instar with 48 h age were used for bioassay. Each experiment was replicated five times.

### *Selection of deltamethrin susceptible strain*

Seven different strains were collected from different locations of wheat growing regions of Pakistan. Ten pairs of adults of each strain were crossed. Bioassays at 6<sup>th</sup> instar larvae were used to identify strains in which the F1 progeny of these crosses were susceptible to deltamethrin. Those in which 100% mortality was observed after 48h, using insecticide concentration equal to LC<sub>20</sub> of the collected strains was then used in second round of crossing. F2 progeny, showing 100% mortality at LC<sub>10</sub> after 48 h in 6<sup>th</sup> instar larvae, was used to propagate the susceptible strain and was designated as S-MDA.

### *Generation of deltamethrin resistant strain*

Fifty 6<sup>th</sup> instar larvae of both male and female of F1 progeny from crosses of ten pairs of collected strains were tested with LC<sub>90</sub> of deltamethrin. The larvae of strains that showed 0% mortality after 48 h at 0.25ppm were reared to adults. The ten pairs of adults were crossed again and 6<sup>th</sup> instar larvae of F2 were exposed to 0.5ppm for 48h. The survivors were reared and ten pairs were crossed. The 6<sup>th</sup> instar larvae of F3 were tested with 1ppm. Larvae that showed 0% mortality were reared. The same procedure was followed by increasing the concentration until F8 when it was tested with 32ppm of deltamethrin. The survivor larvae were reared and the strain was selected to be used as resistant strain in the present study and designated as R-MDA.

### *Toxicant used*

Commercially available formulation of spinosad 240 SC, (Tracer<sup>®</sup>; Dow Agro Sciences) containing 85% spinosyn A and 15% spinosyn D with other spinosyns as minor impurities, was used

in the present study. Acetone was used as solvent for the preparation of different concentrations of the insecticide.

#### *Test of synergism*

The toxicity of spinosad was tested in the presence of synergist, piperonyl butoxide (PBO; Sigma Ltd, UK), an inhibitor of cytochrome P450 monooxygenases (microsomal oxidases) and of esterases. The serial concentrations of spinosad and PBO were prepared using acetone as solvent in the ratios of 1:1, 1:2, 1:4 and 1:8 to test the effect of PBO on the efficacy of spinosad. Mortality data was collected after 48 h when tested against 6<sup>th</sup> instar larvae. The synergism ratio (SR) was calculated by dividing the LC<sub>50</sub> of the strain treated with spinosad alone by the LC<sub>50</sub> of the same strain with spinosad plus synergist.

#### *Contact bioassay*

Series of dilutions of toxicant were prepared using acetone as a solvent. Aliquots of 1 ml of the dilutions were applied into 4.5 cm dia. petridishes for surface-film bioassay (Busvine, 1971). The solvent was allowed to evaporate for 1 hour and larvae transferred to Petri dishes. Controls were treated with acetone alone. Thirty larvae were used for each concentration and the same number of larvae used for control. The Petri dishes were kept in the incubator and mortality observed after 48 h.

#### *Biochemical assays*

All the assays were conducted on 6<sup>th</sup> instar larvae of *T. castaneum* before and after 48 h of treatment by 480ppm spinosad alone and spinosad+PBO in the ratio of 1:4 and the enzyme activity was measured.

#### *Enzyme stock formation*

Larvae (20 to 25) of each strain weighing about 55 mg before and after treatment with spinosad were kept for 2 h at -20°C and ground in mortar pestle under liquid nitrogen to make fine powder. Two hundred micro litres of distilled water was added and the solution centrifuged at 13000 rpm for 10 min. The supernatant as enzyme stock was collected after filtration.

#### *Total protein content*

Total protein content of the enzyme

preparations was measured according to Bradford method (Bradford, 1976). Enzyme activities were expressed in terms of  $\mu\text{mol}/\text{min}$  and presented as specific activities ( $\mu\text{mol}/\text{min}/\text{mg}$  protein).

#### *Catalase assay*

Enzyme dilutions were prepared with 50mM sodium phosphate buffer pH 6.7 for each strain separately and kept in the Eppendorf tubes in the ice-box. Substrate solution was prepared using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and 50mM sodium phosphate buffer. The activity of catalase was measured for 5 min at 240nm in double beam spectrophotometer, Bio Spec-1601 (Shimadzu). The change in absorbance per min was measured and enzyme activity (micromole of H<sub>2</sub>O<sub>2</sub> used per min) was calculated by Beer-Lambert law, using the extinction coefficient of H<sub>2</sub>O<sub>2</sub> as 43M<sup>-1</sup>cm<sup>-1</sup>. Total protein content of crude extract from each species was measured by Bradford method and specific activity of sample was calculated in U/mg.

#### *Amylase assay*

Enzyme dilutions were prepared using 50mM sodium phosphate buffer at pH 7.0 for each strain separately in Eppendorf tubes and kept in ice-box. Dinitrosalicylic acid was mixed in 2M NaOH and slowly added sodium potassium tartarate tetrahydrate as colour reagent. 1% starch was dissolved in 0.05M sodium phosphate buffer by boiling gently. Enzyme dilutions were mixed with dinitrosalicylic acid and starch solution in test tubes and incubated at 25°C for 5 min. The activity of amylase was calculated in double beam spectrophotometer Bio Spec-1601 (Shimadzu), at 540 nm. The specific activity was determined as under:

$$\text{Specific Activity (IU/mg)} = \frac{\text{micromoles maltose liberated}}{\text{mg enzyme used} \times 5\text{min}}$$

One unit is the amount of enzyme which under defined assay conditions will catalyze the conversion of 1.0  $\mu\text{mole}$  of substrate per min.

#### *Acetylcholinesterase (AChE) assay*

The activity of acetylcholinesterase (AChE) was measured according to the method described by Ellman *et al.* (1961). Colour reagent containing

0.25mM Dithiobis, 100mM NaCl and 20mM MgCl<sub>2</sub> was prepared in 50mM Tris-HCl, pH 8. Acetylthiocholine iodide (33mM) was used as substrate. The kinetics of the enzymes reaction was monitored continuously for five min in double beam spectrometer Bio Spec-1601 (Shimadzu), adjusted at 405 nm and 25°C. The specific activity was evaluated by the increase in absorbance after adding 20µL enzyme stock. Absorbance values were converted to units of concentration using a molar extinction coefficient of 13300 M<sup>-1</sup>cm<sup>-1</sup> for acetylthiocholine iodide. The specific activity was expressed as one µmol of acetylthiocholine iodide hydrolysed/min/mL/mg protein at 25°C and pH 8.

#### *Alkaline phosphatase (AkP) assay*

*Para*-nitrophenyl phosphate (*p*NPP) 0.67M was used as substrate. Reaction mixture containing 1mL of 1M diethanolamine buffer mixed in 0.5mM MgCl<sub>2</sub> at pH 9.8 and 17.24µL (*p*NPP), 0.67M was incubated for 5 min at 37°C. After incubation, 20µL of enzyme sample was added and incubated for further 5 min at 37°C. Reaction was stopped by the addition of 1mL of 1M hydrochloric acid (HCl). The released *p*-nitrophenol was determined spectrophotometrically in UTECH (UT1104RS) by measuring the absorbance increase at 405nm using a molar extinction coefficient of 18500M<sup>-1</sup>cm<sup>-1</sup>. One unit of activity represents the amount of enzyme required to produce 1 µmol product per min under the assay conditions. Total protein content was measured by Bradford reagent and specific activity in U/mg of total protein was determined.

#### *Acid phosphatase (AcP) assay*

AcP activity was measured according to the same general principle as that of AkP activity except that this enzyme works at acidic pH 4.8. Reaction mixture containing 1mL of 50mM sodium citrate buffer pH 4.8 along with 5.5 mM *p*-nitrophenyl phosphate and 20µL of enzyme stock was incubated for 30 min in water bath at 37°C and then 1.0 mL of NaOH added to stop the reaction. Increase in absorbance at 405 nm using a molar extinction coefficient of 18500M<sup>-1</sup>cm<sup>-1</sup> was measured against blank using spectrophotometer UTECH (UT1104RS). Total protein content was measured by Bradford reagent method and the specific activity

of AcP was calculated.

#### *Data analysis*

Where necessary, mortality data were corrected using Abbott's formula (Abbott, 1925). The observed data were subjected to probit analysis according to (Finney, 1947) and (Busvine, 1971) using a software Polo-PC to estimate the values of LC<sub>50</sub> and their 95% fiducial limits (FL).

## RESULTS

#### *Susceptibility of S-MDA*

The susceptible S-MDA strain was derived as described earlier. Thirteen strains were collected and their mortality at 6<sup>th</sup> instar larvae in response to 0.125 ppm of deltamethrin was calculated which was found in the range of 0 to 100% after 48 hours (data not shown). The only F1 of collected strains showing 100% mortality was selected for the generation F2, which showed 100% mortality at 0.063 ppm after 48 hours. These were used to start susceptible strain, S-MDA.

Results of bioassays with S-MDA showed that population was significantly susceptible to spinosad as compared to deltamethrin resistant strain; R-MDA followed by R-PHOS, LAB-UK, MUL and FSD strains having very high to slightly higher resistance ratios of 379.93, 6.14, 3.94, 2.52 and 2.46, respectively. All other strains like SWL, KCH, LHR, DGK, CHT, JHG and VRI were found susceptible as they showed non-significant difference in resistance ratios *i.e.* by 1.06, 1.15, 1.21, 1.33, 1.40, 1.43 and 1.87, respectively, against spinosad (Table I).

#### *Response to selection with deltamethrin*

The deltamethrin resistant strain R-MDA was derived as described earlier. The collected thirteen strains were checked for their mortality at 6<sup>th</sup> instar larvae after 48 h in response to 0.25 ppm of deltamethrin (data not shown). The collected strains showing 0% mortality was selected for the generation of resistant strain, R-MDA till F8.

Results of bioassay revealed that R-MDA is highly resistant to spinosad showing very high resistance ratio of 379.93 when compared with S-MDA. Likewise resistance ratios of 358.42, 330.37,

313.99, 285.21, 271.38, 265.69, 203.17, 154.44, 151.01, 96.41 and 61.84 were determined for SWL, KCH, LHR, DGK, CHT, JHG, VRI, FSD, MUL, LAB-UK and R-PHOS, respectively (Table I).

#### Spinosad toxicity

The toxicity of spinosad, in terms of  $LC_{50}$ , after 48 hours of treatment, against 6<sup>th</sup> instar larvae of S-MDA, SWL, KCR, LHR, DGK, CHT, JNG, VRI, FSD, MUL, LAB-UK, R-PHOS and R-MDA strains, was 609, 648.2, 697.5, 737.9, 809.6, 851.3, 872.8, 1140.7, 1500.6, 1532.2, 2400, 3741.3 and 231375.2 ppm, respectively. No mortality was observed in control experiment (Table I).

#### Effect of enzyme inhibitor (PBO) on resistance

The effect of microsomal oxidase inhibitor, PBO on the efficacy of spinosad was tested in different ratios against MUL, LHR, FSD and R-MDA strains (Tables II-V). The results revealed that spinosad was found to be the most effective insecticide against MUL and FSD strains (slightly resistant to deltamethrin) when treated in 1:2 with PBO. In case of LHR strain (susceptible to deltamethrin) its efficacy was highest in 1:1 with PBO where as it was proved to be the most toxic insecticide when used in 1:4 with PBO against R-MDA strain (highly resistant to deltamethrin).

#### Total protein

The total protein content in 6<sup>th</sup> instar larvae of field strains was significantly ( $p < 0.05$ ) higher after the treatment with the sub-lethal dose of spinosad than that of untreated. All field strains; DGK, SWL, LHR, KCH, FSD and MUL showed 41.61%, 31.56%, 23.25%, 79.39%, 70.40% and 212.47% increase, respectively. On the other hand 17.85%, 26.92% and 68.36% decrease in the total protein content was recorded in all lab strains; R-PHOS, LAB-UK and R-MDA, respectively.

When comparing the total protein content in untreated with that of spinosad+PBO in the ratio 1:4, there was 15.12%, 4.86%, 14.94%, and 6.28%, decrease in DGK, LHR, KCH, and FSD, respectively. An increase in the total protein content of 6.4% and 56.91% was recorded in SWL and MUL, respectively. In case of lab strains, a decrease of 17.85%, 32.81% and 82.85% was observed in R-

PHOS, LAB-UK and R-MDA, respectively.

The results revealed decrease of total protein content in all the strains when spinosad+PBO treated was compared with that of spinosad treated at sub lethal dose. Field and lab strains *i.e.* DGK, SWL, LHR, KCH, FSD, MUL, LAB-UK and R-MDA exhibited 40.06%, 19.12%, 22.81%, 52.59%, 45.00%, 49.78%, 8.06% and 45.80%, decrease, respectively. The total protein content in R-PHOS strain after both the treatments was same (Fig. 1).

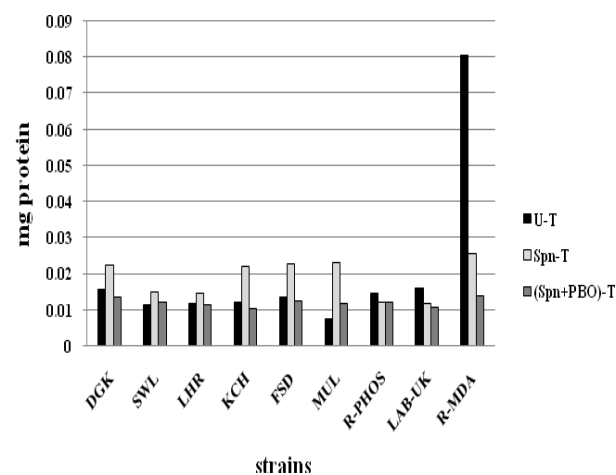


Fig. 1. Total protein (mg) in 6<sup>th</sup> instar larvae of field (susceptible) and lab (resistant) strains for untreated, spinosad (480ppm) and spinosad+PBO treatments. DGK, SWL, LHR, KCH, FSD and MUL, field (susceptible) strains and R-PHOS, LAB-UK and R-MDA, lab (resistant) strains U-T, Untreated; Spn-T, spinosad treated; Spn+PBO-T, spinosad+PBO treated in 1:4.

#### Effect on enzyme activity

##### Catalase

The results revealed that specific activity of catalase in the 6<sup>th</sup> instar larvae of all the field as well as lab strains was significantly higher by 2235.82%, 2456.82%, 2586.42%, 363.22%, 1321.97%, 2115.96%, 1045.21%, 1292.29% and 5431.38% in DGK, SWL, LHR, KCH, FSD, MUL, R-PHOS, LAB-UK and R-MDA, respectively after the treatment of sub lethal dose of spinosad than that of untreated.

Similar results were obtained in both, field and lab strains when specific activity of catalase after the treatment of spinosad + PBO (1:4) was

**Table I.- Response (mortality) of 6<sup>th</sup> instar larvae of *Tribolium castaneum* against spinosad.**

Strains	LC <sub>50</sub> (95%FL) (ppm)	Fit of probit line						
		Slope (± SE)	χ <sup>2</sup>	df	p	n <sup>a</sup>	RR <sup>b</sup>	RR <sup>c</sup>
DGK	809.6 (566.5-1329.7)	1.399 (±0.27)	0.40	3	0.94	150	1.33	285.21
MUL	1532.2 (1055.0-2937.7)	1.637 (±0.32)	0.02	3	0.99	150	2.52	151.01
VRI	1140.7 (812.7-1886.4)	1.555 (±0.29)	0.17	3	0.98	150	1.87	203.17
FSD	1500.6 (910.1-4316.8)	1.070 (±0.27)	0.44	3	0.93	150	2.46	154.44
CHT	851.3 (600.8-1347.2)	1.431 (±0.28)	0.40	3	0.94	150	1.40	271.38
JHG	872.8 (605.9-1484.0)	1.377 (±0.27)	0.26	3	0.97	150	1.43	265.69
LHR	737.9 (501.8-1197.0)	1.281 (±0.27)	0.08	3	0.99	150	1.21	313.99
SWL	648.2 (420.5-1077.6)	1.162 (±0.26)	0.55	3	0.90	150	1.06	358.42
KCH	697.5 (456.1-1184.3)	1.165 (±0.26)	0.97	3	0.81	150	1.15	330.37
LAB-UK	2400.0 (1725.9-3679.7)	1.528 (±0.28)	1.13	3	0.77	150	3.94	96.41
R-PHOS	3741.3 (2620.8-6611.9)	1.552 (±0.29)	0.05	3	0.99	150	6.14	61.84
S-MDA	609.0 (437.5-867.8)	1.562 (±0.28)	0.16	3	0.98	150	-	379.93
R-MDA	231375.2 (169748.9-342770.9)	1.649 (±0.29)	0.44	3	0.94	150	379.93	-

<sup>a</sup>Number of larvae used in bioassay, including control<sup>b</sup>Resistance ratio = LC<sub>50</sub> of collected strains / LC<sub>50</sub> of S-MDA strains<sup>c</sup>Resistance ratio = LC<sub>50</sub> of R-MDA strains / LC<sub>50</sub> of collected strains**Table II.- Response of 6<sup>th</sup> instar larvae of MUL strain *Tribolium castaneum* against different ratios of spinosad+PBO**

Strains	Ratios Spin : PBO	LC <sub>50</sub> (95%FL) (ppm)	Fit of probit line				
			Slope (± SE)	χ <sup>2</sup>	df	p	n
MUL	1 : 1	1370.4 (702.2-9090.8)	1.061 (±0.29)	0.15	3	0.99	150
MUL	1 : 2	601.8 (400.9-1093.6)	1.204 (±0.27)	0.38	3	0.94	150
MUL	1 : 4	616.3 (436.1-877.6)	1.520 (±0.27)	0.32	3	0.96	150
MUL	1 : 8	613.0 (405.6-837.3)	1.654 (±0.29)	0.33	3	0.95	150

N, Number of larvae used in bioassay, including control

**Table III.- Response of 6<sup>th</sup> instar larvae of LHR strain *Tribolium castaneum* against different ratios of spinosad+PBO**

Strains	Ratios Spin : PBO	LC <sub>50</sub> (95%FL) (ppm)	Fit of probit line				
			Slope (± SE)	χ <sup>2</sup>	df	p	n
LHR	1 : 1	78.0 (38.5-115.9)	1.488 (±0.30)	1.49	3	0.68	150
LHR	1 : 2	246.7 (122.2-400.2)	1.003 (±0.26)	0.10	3	0.99	150
LHR	1 : 4	229.9 (131.6-325.3)	1.624 (±0.31)	0.29	3	0.96	150
LHR	1 : 8	724.5 (328.3-1212.3)	0.940 (±0.25)	0.04	3	0.99	150

n, Number of larvae used in bioassay, including control

**Table IV.- Response of 6<sup>th</sup> instar larvae of FSD strain *Tribolium castaneum* against different ratios of spinosad+PBO**

Strains	Ratios Spin : PBO	LC <sub>50</sub> (95%FL) (ppm)	Fit of probit line				
			Slope (± SE)	χ <sup>2</sup>	df	p	n
FSD	1 : 1	326.9 (232.5-494.8)	1.468 (±0.27)	1.01	3	0.79	150
FSD	1 : 2	245.6 (162.5-342.9)	1.503 (±0.28)	1.95	3	0.58	150
FSD	1 : 4	526.5 (406.2-668.6)	2.305 (±0.34)	2.41	3	0.49	150
FSD	1 : 8	1206.7 (784.3-1931.6)	1.194 (±0.26)	0.23	3	0.97	150

n, Number of larvae used in bioassay, including control

**Table V.-** Response of 6<sup>th</sup> instar larvae of R-MDA strain *Tribolium castaneum* against different ratios of spinosad+PBO.

Strains	Ratios		LC <sub>50</sub> (95%FL) (ppm)	Fit of probit line				
	Spin : PBO			Slope ( $\pm$ SE)	$\chi^2$	df	p	n
R-MDA	1 : 1		646.5 (424.3-1385.6)	1.304 ( $\pm$ 0.28)	0.12	3	0.98	150
R-MDA	1 : 2		485.7 (353.1-706.7)	1.589 ( $\pm$ 0.28)	0.19	3	0.98	150
R-MDA	1 : 4		305.4 (209.2-405.7)	1.892 ( $\pm$ 0.32)	2.88	3	0.41	150
R-MDA	1 : 8		452.4 (309.6-593.6)	2.118 ( $\pm$ 0.36)	1.61	3	0.66	150

n, Number of larvae used in bioassay, including control

compared with that of untreated larvae showing 2075.14%, 2206.91%, 2422.47%, 313.97%, 1083.23%, 1213.33%, 306.26%, 892.15% and 2330.06% highly significant increase in DGK, SWL, LHR, KCH, FSD, MUL, R-PHOS, LAB-UK and R-MDA, respectively.

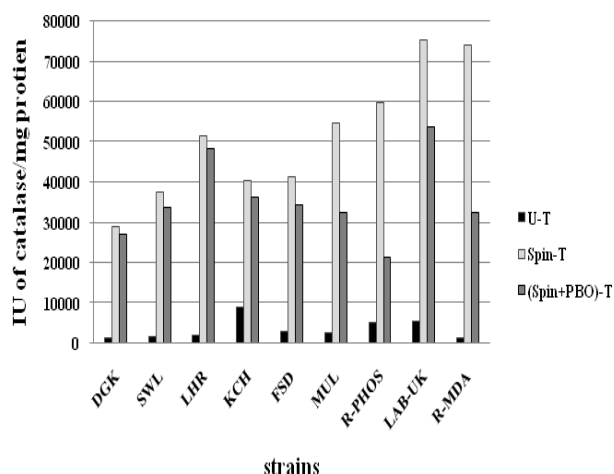


Fig. 2. Specific activity of catalase (IU/mg of protein) in 6<sup>th</sup> instar larvae of field (susceptible) and lab (resistant) strains for untreated, spinosad (480ppm) and spinosad+PBO treatments. For explanation of other abbreviations see Figure 1.

A decrease of 6.88%, 9.77%, 6.10%, 10.63%, 16.79%, 40.73%, 64.53%, 28.74%, and 56.07% in the levels of specific activity of catalase was observed in DGK, SWL, LHR, KCH, FSD, MUL, R-PHOS, LAB-UK and R-MDA strains, respectively when spinosad+PBO treated larvae were compared with that of spinosad treated at sub lethal dose.

### Amylase

The specific activity of amylase was decreased in the field strains *i.e.* DGK, SWL, KCH, FSD and MUL by 12.97%, 21.62%, 34.01%, 42.52% and 38.99%, respectively and 30.61% increase in LHR strain. An increase of 3.63%, 77.74% and 494.59% in the level of amylase was observed in lab strains, R-PHOS, LAB-UK and R-MDA, respectively when compared with that of untreated larvae.

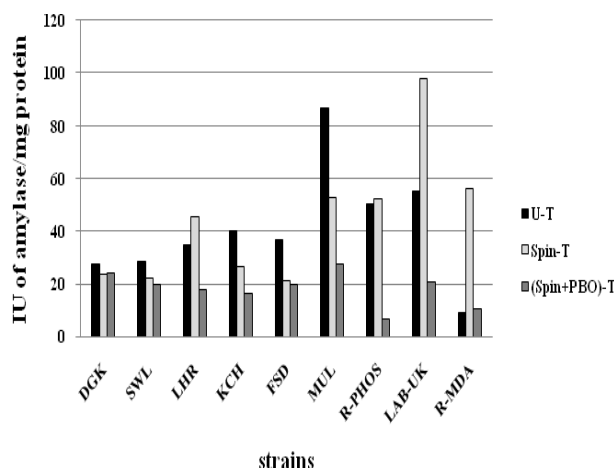
The results also revealed a decrease in the levels of amylase in both field as well as lab strains by 12.32%, 29.50%, 48.71%, 59.20%, 45.58%, 68.14%, 86.85% and 62.35% in DGK, SWL, LHR, KCH, FSD, MUL, R-PHOS and LAB-UK, respectively. On the other hand an increase of 13.08% was observed in R-MDA when spinosad+PBO (1:4) treatment was compared with that of control.

When comparison of spinosad treatment at sub lethal dose was made with spinosad+PBO (1:4) treatment, the results revealed that 6<sup>th</sup> instar larvae of field strains, SWL, LHR, KCH, FSD and MUL showed a decrease of 10.05%, 60.73%, 38.16%, 5.32% and 47.78%, respectively and non significant increase of 0.75% in DGK. Conversely, R-PHOS LAB-UK and R-MDA lab strains revealed a decrease of 87.31%, 78.82% and 80.65%, respectively in the levels of specific activity of amylase.

### Acid phosphatase

The results of specific activity levels of AcP between larvae of spinosad treatment and control showed 100% and 11.11% increase in SWL and KCH respectively. Other field strains, LHR, FSD and MUL showed 4.55%, 30.43% and 50.00% decrease, respectively. DGK showed neither

increase nor decrease. In case of lab strains, R-PHOS revealed 58.33% decrease whereas LAB-UK and R-MDA showed 5.56% and 70.00% increase, respectively.



strains  
Fig. 3. Specific activity of amylase (IU/mg of protein) in 6<sup>th</sup> instar larvae of field (susceptible) and lab (resistant) starins for untreated, spinosad (480ppm) and spinosad+PBO treatments  
For explanation of other abbreviations see Figure 1.

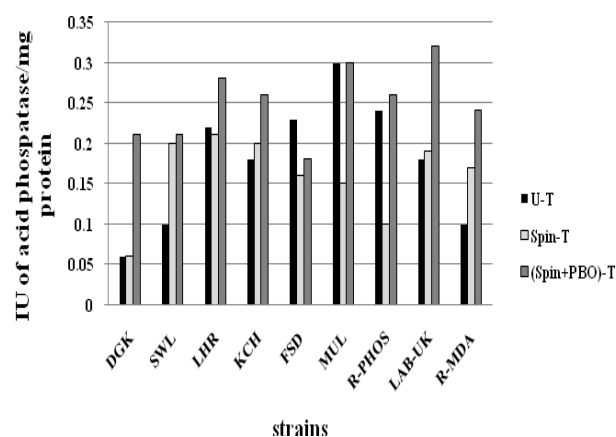
A significant increase in the levels of AcP was observed when larvae treated with spinosad+PBO (1:4) were compared with that of control showing 250%, 110.00%, 27.27% and 44.44% increase in field strains, DGK, SWL, LHR and FSD, respectively. A slight decrease of 21.74% was observed in FSD whereas MUL showed non-significant changes. All lab strains *viz.* R-PHOS, LAB-UK and R-MDA showed increase of 8.33%, 77.78% and 140.00%, respectively.

Larvae of all field strains such as DGK, SWL, LHR, KCH, FSD and MUL exhibited 250.00%, 5.00%, 33.33%, 30.00%, 12.50% and 100.00% increase, respectively in the specific activity of AcP when spinosad+PBO (1:4) treated were compared with that of spinosad treated at sub lethal dose. Similar results were obtained in all lab strains, R-PHOS, LAB-UK and R-MDA by showing 160.00%, 68.42% and 41.18% increase, respectively.

#### Alkaline phosphatase

When comparison was made between

spinosad treatment and control, the results revealed that all field strains, DGK, SWL, KCH, FSD and MUL showed decrease of 23.53%, 40.00%, 83.33%, 52.38% and 38.78% in the specific activity levels of AkP, respectively. LHR exhibited an increase of 21.43% in AkP activity. In case of lab strains of R-PHOS and LAB-UK, a respective decrease of 49.32% and 81.71% was observed. Conversely R-MDA showed a significant increase of 633.33% in AcP activity.



strains  
Fig. 4. Specific activity of acid phosphatase (IU/mg of protein) in 6<sup>th</sup> instar larvae of field (susceptible) and lab (resistant) starins for untreated, spinosad (480ppm) and spinosad+PBO treatments  
For explanation of other abbreviations see Figure 1.

The results revealed an increase in AkP levels of 170.59%, 144.00%, 114.29% and 71.43% in DGK, SWL, LHR and FSD field strains, respectively when spinosad+PBO (1:4) treatment was compared with that of control. KCH and MUL exhibited a decrease in levels of AkP by 63.33% and 22.45%, respectively. Likewise an increase of 21.95% and 933.33% was observed in LAB-UK and R-MDA lab strains, respectively. A non-significant change in AkP level was observed in R-PHOS strain.

The results exhibited an increase in the specific activity levels of AkP in all field as well as lab strains when spinosad+PBO (1:4) treatment was compared to spinosad treatment. AkP levels in all field strains *i.e.* DGK, SWL, LHR, KCH, FSD and



MUL were increased by 253.85%, 306.67%, 76.47%, 120.00%, 260.00% and 26.67%, respectively. Similarly all lab strains viz. R-PHOS, LAB-UK and R-MDA also exhibited an increase of 97.30%, 566.67% and 40.91%, respectively in AkP levels.

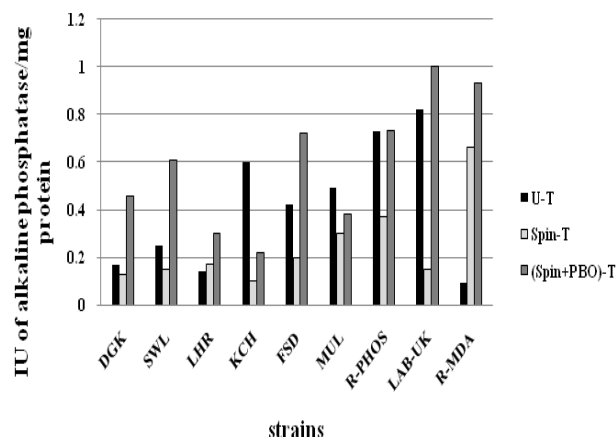


Fig. 5. Specific activity of alkaline phosphatase (IU/mg of protein) in 6<sup>th</sup> instar larvae of field (susceptible) and lab (resistant) strains for untreated, spinosad (480ppm) and spinosad+PBO treatments. For explanation of other abbreviations see Figure 1.

#### Acetylcholinesterase (AChE)

The results revealed that specific activity of AChE in the 6<sup>th</sup> instar larvae of all the field strains, DGK, SWL, LHR, KCH, FSD, MUL, as well as lab strains, R-PHOS, LAB-UK and R-MDA was significantly higher by 141.52%, 129.20%, 190.32%, 77.65%, 101.32%, 35.16%, 354.17%, 1213.95% and 34867.39%, respectively after the treatment of sub lethal dose of spinosad than that of untreated.

A significant increase in the levels of AChE was observed when larvae treated with spinosad+PBO (1:4) were compared with that of control showing 216.37%, 109.40%, 49.31%, 214.53%, 147.58% and 81.64% increase in DGK, SWL, LHR, KCH, FSD and MUL field strains, respectively. Similarly 222.92%, 1026.74% and 5791.30% increase in the AChE levels was found in R-PHOS, LAB-UK and R-MDA lab strains, respectively.

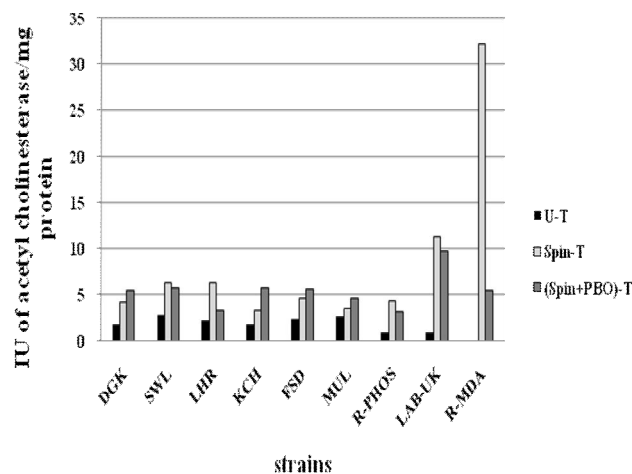


Fig. 6. Specific activity of acetyl cholinesterase (IU/mg of protein) in 6<sup>th</sup> instar larvae of field (susceptible) and lab (resistant) strains for untreated, spinosad (480ppm) and spinosad+PBO treatments. For explanation of other abbreviations see Figure 1.

The specific activity levels of AChE were increased by 30.99%, 77.04%, 22.98% and 34.39% in field strains *i.e.* DGK, KCH, FSD and MUL after the treatment with spinosad+PBO (1:4). Contrary a decrease of 8.60% and 48.57% was recorded in SWL and LHR strains when compared with spinosad treatment. Similarly a respective decrease of 28.90%, 14.25% and 83.15% was observed in specific activity levels of AChE after the treatment with spinosad+PBO as compared to spinosad treatment, in LAB-UK, R-PHOS and R-MDA lab strains.

## DISCUSSION

The results of present experimentation revealed that field strains of *T. castaneum* were susceptible to spinosad than lab strains. It could be due to the reason that at the time of insect collection; (i) there was a shortage of wheat in the country, (ii) most of the government stores had fresh stocks and (iii) deltamethrin and spinosad were not used for grain treatment in Pakistan. Our findings are consistent with those reported by Norma and Morallo-Rejesus, (1981) that strains of *T. castaneum*

tested for resistance to pirimiphos methyl, showed a susceptible response to this insecticide. It was found that at the date of collection pirimiphos methyl had been used to control these storage insects in 37 stores during the previous five months, and had not been used in the remaining stores. Five months is probably too short a time for *T. castaneum* to develop resistance to pirimiphos methyl. This would probably explain the susceptible response of all the strains to pirimiphos methyl.

Our findings revealed different degrees of susceptibility in field strains of *T. castaneum*, which was not of practical significance. Larvae were less susceptible to spinosad showing least LC<sub>50</sub> value (648.2ppm) for SWL strain and highest LC<sub>50</sub> value (1532.2ppm) for MUL strain. Our results are in full agreement with Norma and Morallo-Rejesus (1981), who reported that strains of *T. castaneum* obtained from farmers' stores at different places, showed varying degrees of resistance despite the fact that these stores had no previous insecticidal operations. This could be explained to the fact that *T. castaneum* are strong and frequent fliers and are thus able to migrate actively, and that they move passively with the stored commodities. Much evidence in the literature showed that resistant strains of storage insects were often found where the insecticides had not been used (Champ and Brown, 1970; Pieterse *et al.*, 1972). Similarly, Subramanyam *et al.* (1989) reported that four field strains of the sawtoothed grain beetle, *Oryzaephilus urinamensis* (L.), collected from farm-stored barley, were less susceptible to the grain protectant chlorpyrifos-methyl, compared with a laboratory strain. Stored barley from which the four field strains of *O. surinamensis* were collected had not been treated with chlorpyrifos-methyl. Fang *et al.* (2002a) and Flinn *et al.* (2004) showed that *T. castaneum* adults were less susceptible to spinosad. The rate of 1 mg/kg is effective in preventing progeny production in laboratory and field trials because larvae hatching from the eggs were highly susceptible to spinosad.

Selection of the field population with deltamethrin significantly, (almost 380 times) increased its resistance against spinosad from LC<sub>50</sub> of 609ppm to LC<sub>50</sub> of 231375.2ppm. This showed cross resistance in *T. castaneum*. It has also been

reported by other researchers. For example, the malathion-resistant strains showed cross-resistance to bromophos, (Pieterse *et al.*, 1972). Speirs and Zettler (1968) reported that malathion-resistant beetles showed resistance to pyrethrins and other conventional insecticides. This could be related to the phenomena that insect may use more than one resistant mechanism (multiple resistances) or the same mechanism may confer resistance to more than one compound or group of compounds. For instance, carboxylesterases catalyse the hydrolysis of a wide range of xenobiotic carboxylesters and aromatic amides and is involved in a major route for the detoxification and the activation of such compounds (Ketterman *et al.*, 1992) and subsequently leads to insecticide resistance (Oppenoorth, 1985). The most commonly observed change that has been linked to resistance development is the increased carboxylesterase activity (Raymond *et al.*, 1986). The putative mechanism involves a gene amplification that results in an overproduction of an esterase in resistant insects (Mouches *et al.*, 1986; Field *et al.*, 1988). Kinetic properties of this esterase are similar in susceptible and resistant insects (Devonshire, 1977); the esterase hydrolyses insecticides very slowly, but in highly resistant strains the enzyme accounts for 12% of the total soluble protein (Mouches *et al.*, 1986). The esterase abundance apparently protects the insects by binding and sequestering insecticides rather than by rapid hydrolysis.

The slopes of the dose-mortality curves at S-MDA and R-MDA were not significantly different, which indicated low genetic variation between both the strains. It has been reported by Hoskins (1960) that the slope of the dose-mortality curves represent the phenotypic variation in susceptibility in the population comprising both environmental and genetic components. However, Chilcutt and Tabashnik (1995) suggest that the slope of the concentration-mortality line is not a good indicator of genetic variation in susceptibility owing to being confounded by the environmental component of variation.

In all susceptible field strains, the total protein content was lower before the treatment with sub lethal dose of spinosad. Contrary it was higher

in case of two resistant lab strains, R-PHOS and LAB-UK while significantly higher in R-MDA strain. After the treatment with sub lethal dose of spinosad, the results were reversed *i.e.* all field strains had a higher level of total protein but its level was decreased in all resistant lab strains and showed highly significant decrease in R-MDA. Similarly the total protein contents after the treatment with spinosad+PBO (1:4) remained at considerably low levels in all susceptible field as well as resistant lab strains. Our findings are in partial conformity with that of Hussain *et al.* (2009) who reported that spinosad treatment decreased the total protein contents in adult beetles of *T. castaneum*. It showed utilization of protein in energy production. In contrast Saleem *et al.* (2001) reported that gamma-HCH, at LC<sub>10</sub> (10 mg L<sup>-1</sup>) and LC<sub>20</sub> (20 mg L<sup>-1</sup>) decreased the total protein contents in adult beetles of *T. castaneum*. Bifenthrin, more prominently changed the total protein contents, in FSS-II strain, than in PAK strain after the treatment with a sublethal dose (200 mg L<sup>-1</sup>) (Shakoori *et al.*, 1994). The decline in proteins level with increase in transaminase activities suggests the mobilization of amino acids during insecticide stress to meet the energy demands.

Our findings revealed that spinosad+PBO mixture is effective in ratios of 1:1 and 1:2 against relatively less resistant strains and most effective when used in 1:4 against highly resistant strain to deltamethrin. A highly significant decrease in LC<sub>50</sub> of spinosad, from 231375.2 ppm to 305.4 ppm was observed when used in mixture with PBO. Synergistic effect gradually decreased after 1:4 as the ratios increased. Our studies are partially in line with Rahman *et al.* (2007) who reported that PBO synergized both malathion and lambda cyhalothrin at 1:1 ratio and greater. Moreover, the synergism increased with the increase of PBO in the mixture. Sun and Johnson (1960) suggested that the synergistic effect of sesamex and related compound was due to the inhibition of biological oxidation. The same mechanism of action may also occur to the combinations in our studies. The synergistic action of some pyrethroid synergists were studied with malathion by Rai *et al.* (1956) and Ware and Roan (1958). Synergistic effects of PBO in combination with several insecticides were studied

by Hadaway *et al.* (1962) and Bengston *et al.* (1983). The effect of PBO on toxicity of insects has been studied by several scientists. It is now generally well documented that PBO produces its synergistic effect by inhabiting the detoxification enzymes within the insect body (Casida 1970; Benke and Wilkinson, 1971).

The physiological function of catalase, an enzyme which reduces hydrogen peroxide to water and oxygen is largely unknown, but there is an evidence to suggest that it plays a vital part in the detoxification of free radicals derived from oxygen (Masters and Holmes, 1979). Our results showed that specific activity of catalase in the 6<sup>th</sup> instar larvae of all field (susceptible) as well as lab (resistant) strains was significantly higher after the treatment of both, spinosad alone and spinosad+PBO (1:4) than that of untreated. A decrease in the levels of specific activity of catalase was observed in all the strains when spinosad+PBO treated were compared with that of spinosad treated at sub lethal dose which confirms that PBO works as catalase inhibitor. Nicholas *et al.* (1982) reported that catalase may play a role in the toxicity of phosphine to insects and there is preliminary evidence that treatments which raise catalase levels increase the tolerance of insects to phosphine.

Our studies revealed that specific activity of amylase was at lower levels in most of the spinosad and spinosad+PBO treated field strains. Conversely in lab strains it was at significantly higher level compared with that of untreated larvae. Our results are in partial agreement with Nehad *et al.* (2008) reported that activities of the carbohydrate hydrolyzing enzymes (amylase, trehalase and invertase) were decreased after 24 hours post treatment. Moreover, Saleem and Shakoori (1987a) reported that pyrethroids at sub-lethal concentrations had shown a decreased gut amylase activity in *T. castaneum* larvae. Increased level of amylase in lab strains, indicate more carbohydrate utilization so that more energy can be obtained to combat insecticidal stress. In comparison of two treatments *i.e.* spinosad alone and spinosad+PBO, results revealed that larvae of field as well as lab strains showed a decrease in the levels of specific activity of amylase. Efficient amylase must be essential for the survival of flour beetles. They live

in flour and other starch containing commodities. Amylolysis of starch in *T. castaneum* is mainly due to amylase. Reduction in amylase level may be life threatening. We can conclude that PBO acts as amylase inhibitor in both susceptible and resistant *T. castaneum* larvae.

We observed no regular pattern in specific activity levels of phosphatases (AcP, AkP) before and after the treatment with spinosad in all field strains. Similar results were obtained in lab strains. Likewise no particular pattern was observed when larvae treated with spinosad+PBO (1:4) were compared with that of control. Conversely all lab strains showed increase in the levels of both phosphatases (AcP, AkP). In the same way larvae of all field and lab strains exhibited increase in the specific activity of phosphatases (AcP, AkP) when spinosad+PBO (1:4) treated larvae were compared with that of spinosad treated. It confirms that PBO does not work as synergist in case of this enzyme. Our findings are in full agreement with Hussain *et al.* (2009) who reported that spinosad caused changes in the activities of phosphatases (AcP, AkP), in adult beetles of both the strains, but did not produce any pattern. Many other scientists discovered that any change in the activities of these important enzymes, may affect the glucose phosphorylation, breakdown of ATP, the digestion of phospholipids and growth rate (Saleem and Shakoori, 1987).

AChE is an enzyme that occurs in the central nervous system. It functions by removing acetylcholine from its postsynaptic receptor. The result of this action is the hydrolysis of acetylcholine into acetate and prolonged neuroexcitation. Our studies revealed that specific activity of AChE in the larvae of all the field as well as lab strains was significantly higher after the spinosad and spinosad+PBO (1:4) treatments than that of untreated. Our results are in full agreement with Hassan *et al.* (2009) who reported that activity of AChE was increased in *Spodoptera littoralis* (Biosd) larvae after the treatment of methylamine avermectin. This increase of AChE activity could refer to the new mode of action of this newly derived avermectins, which seem to work in a similar manner of other closely related compound (*i.e.*, metabolites of actinomycetes). Comparing the

specific activity levels of AChE between larvae of spinosad and spinosad+PBO (1:4) treated, the results revealed an increase and decrease in some field strains. On the other hand a decrease in the specific activity levels of AChE was observed in lab strains, it confirms that PBO inhibits the AChE especially in resistant strains.

From the results described above it can be concluded that spinosad alone can be more effective against less resistant strains. Its mixture with PBO in the ratio of 1:4 is fairly effective against resistant strains because PBO is an established enzyme inhibitor. Spinosad+PBO mixture inhibits catalase, amylase, phosphatases and acetylcholinesterase enzymes especially in resistant strains. Further studies are needed more precisely at molecular level to strictly detect the mode of action of this newly developed compound which holds much promise to control insects due to its novel mode of action.

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