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RESEARCH ARTICLE

BIOCHEMICAL BASIS OF INSECTICIDE RESISTANCE IN THE WHITE BACKED PLANT HOPPER, *SOGATELLA FURCIFERA* (HORVATH) INFESTING RICE

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ABSTRACT

The whitebacked planthopper, (*Sogatella furcifera* (Horvath) is a major threat to rice crops throughout Asia, damaging plants both through its feeding behavior and by acting as a vector. Due to high insecticide pressure, it is becoming resistant to many insecticides. Many enzymes are known to be involved in providing resistance to these planthoppers against insecticides. Fewer research investigations have been conducted on insecticide resistance in relation to WBPH The objective of this paper was to evaluate the biochemical basis of resistance probably by evolving new isoforms of insecticide detoxifying enzymes. Biochemical profile of esterase, acetylcholinesterase and glutathione S-transferase (GST) was assessed. No difference in the glutathione-S-transferase activity was seen in buprofezin and quinalphos, however significant increase in GST activity was seen in chlorntraniliprole resistant insects. Buprofezin was highly toxic as compared to quinalphos and chlorntraniliprole. Esterase (30.82 µg of napthol formed/min/mg of insect) and acetylcholinesterase (17.23 µg of glutathione formed/min/mg of insect activity) was higher in quinalphos. The results indicate the potential development of esterases as detoxification mechanisms responsible for resistance to buprofezin and quinalphos. Esterase isozyme profiling using native PAGE shows dark bands in Buprofezin and quinalphos however diffused light bands were observed in case of chlorntraniliprole, indicate GST as the principal enzyme for detoxification of chlorntraniliprole.

INTRODUCTION

Rice planthoppers especially the whitebacked planthopper (WBPH), *Sogatella furcifera* (Horvath) is the major delphacid hopper attacking rice, *Oryza sativa* L. in tropical Asia (Matsumara *et al.*, 2009). Hopper-burn caused by planthoppers threatens global rice crops, particularly in Asia (Backus *et al.*, 2005). WBPH also has recently been reported to transmit the fijivirus, Rice black stark dwarf virus -2 (RBSDV-2) (Zhang *et al.*, 2008) and the southern rice black streaked dwarf virus (SRBSDV) (Zhou *et al.*, 2008) in hybrid rice is a matter of concern in rice growing countries. Insecticides have been increasingly used to control WBPH. Due to continuous exposure to insecticides, insects generally develop resistance to these chemicals (Armes *et al.*, 1992; Kranthi *et al.*, 2002). National Agricultural Research and Extension Systems (NARES) are looking to host-plant resistance as a possible solution to these outbreaks (Brar *et al.*, 2009). Insecticide resistance is mainly attributed to two main mechanisms at the molecular level (1) the increased production of metabolic enymes (esterases, glutathione S transferases, and cytochrome P450 monooxygenases), which can break down or bind to the pesticide and (2) structural changes in the pesticide target protein, which render it less sensitive to the toxic effects of the pesticide (Bass and Field, 2011). Acetylcholinesterase (AchE) is a key enzyme in insect nervous system, which terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter, acetylcholine and alteration of AchE plays a role to contribute resistance to insecticides (Yang *et al.*, 2008). The increased production of glutathione-S-transferase (GST) has been documented as a mechanism of resistance to organochlorines and pyrethroids which is most commonly mediated through its upregulation (Li *et al.*, 2007). One or more GST isoforms have been found to be associated with resistant strains of planthopper, *Nilaparvata lugens* (Vontas *et al.*, 2001, 2002). Fewer research investigations have been conducted on insecticide resistance in relation to WBPH (Nagata, 2002, Ling *et al.*, 2009). Lopez-Soler *et al.* 2008 reported increase in number of esterase bands in resistant strains in comparison to susceptible ones. A significant correlation between esterase activity and resistance to acrinathrin and methiocarb was observed (Myamo *et al.*, 2006). The objective of the present study was to derermine the current status of resistance of WBPH to buprofezin, quinalphos and chlorntraniliprole in terms of activities of enzymes esterase, acetylcholinesterase and GST.

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MATERIALS AND METHODS

Test insect; The fifth instar nymphs of *S.furcifera* were collected from the WBPH culture already maintained for over

eight generations at Entomological research farm of PAU and maintained separately at desired temperature and relative humidity conditions in screen house. The collected insects were reared on one month old rice plants of susceptible rice variety, TN1 sown in pots. The pots were placed in a water tray (70 × 50 × 12 cm) and covered with steel bar frame cage (68 × 48 × 50 cm) surrounded with nylon nett (40 mesh) from all around and the top. The hopper adults were then released in the cage using an aspirator, for multiplication. The plants that dried away due to insect feeding, were replaced with fresh potted plants of the same age. Two to three such cages of insect culture were generally maintained throughout the study period to procure sufficient number of the test insects.

Test insecticides and bioassays

The commercial formulations of insecticides *viz.* chlorntraniliprole, buprofezin, quinalphos were used. At least eight concentrations for each insecticide were used in bioassays. (Table 1).

Table 1. Common and tradename of insecticides

S. No.	Common name	Tradename
1	Chlorntraniliprole	Coragen
2	Buprofezin	Applaud
3	Quinalphos	Ekalux

For various bioassay experiments, 30-35 days old seedlings of the susceptible rice variety, TN1 were used. These seedlings were pulled out of pots along with roots and washed thoroughly. The stems were cut 10 cm above the root and air-dried to remove excess water. These rice stems were grouped and dipped into appropriate insecticide dilutions for 30 s. Three replicates were maintained for each dose of insecticides along with a water treated control. After drying for few minutes, rice stems were placed in 500 ml plastic cups with root portion placed in a mixture of vermicompost, sand and water. Fifteen fifth instar nymphs were released into each plastic cup using an aspirator and retained in the cup using muslin cloth tied with rubber band and maintained at room temperature. Mortality was recorded up to 4 days after the treatment and LC_{50} was determined by probit analysis. The fifth in star nymphs were exposed to insecticides at concentrations corresponding to the LC_{50}. After eight generation of insecticide selection pressure the insects were used for biochemical assays. Different biochemical assays were used to standardize and study the activity of Acetylcholine esterase, Glutathione-S- Transferase and Esterase.

Enzyme Assay

Acetylcholine esterase (AchE) extraction: (Ellman *et al.* 1961)

AchE was prepared by homogenising 50 insect heads in 1.5 ml of ice-cold 0.1M phosphate buffer (pH 7.5) containing 0.3% (v/v) Triton-X-100. The homogenate were centrifuged at 10,000g for 20minutes at 4°C. The supernatant thus obtained was used as enzyme extract for determination of enzyme activity.

Enzyme Assay: 20µl of homogenate was used for initiating reaction in a reaction mixture containing 1.6ml of phosphate buffer, 0.1 ml of acetylthiocholine iodide and 0.1 ml of DTNB and was incubated for 30 min at 37°C. The reaction was stopped with 0.1 ml of ethion. The absorbance was measured at 405 nm against reagent blank after 10 min. The enzyme activity was calculated from standard curve of reduced glutathione reduced (6 - 62.5µl) and was expressed in the form of µg of free thiol group formed/min/mg of insect.

Esterase (Wool and Greenburg, 1990)

Extraction: Fifty insects were homogenized in pre-chilled Teflon homogenizer using 3ml of ice-cold 0.1 M phosphate buffer (pH 6.5, having 0.1% Triton X-100). The extract was centrifuged at 10,000g for 20min. The supernatant thus obtained was used as enzyme extract for determination of enzyme activity. All these operations were carried out at 4°C.

Enzyme Assay: The enzymatic reaction was initiated by adding 0.05ml of homogenate in reaction mixture, 0.2 ml substrate solution and 1.7 ml of phosphate buffer and was incubated for 30 min at 37°C. The reaction was stopped and color was developed by adding 0.5ml of 0.3% Fast Blue B solution. Read the absorbance at 602 nm against reagent blank, containing phosphate buffer substrate solution and Fast Blue-B-solution after 10 min. The enzyme activity was calculated from a standard curve prepared by using different concentrations (2.5 – 15 µl) of alpha- napthol. The enzyme activity was expressed in the form of µg of napthol formed/min/mg of insect.

Glutathione-S-Transferase (GST) Habig *et al* (1974)

The method developed by Habig was adapted for measuring GST activity, fifty insects were homogenized in pre-chilled Teflon homogenizer using 3ml of ice-cold 0.1 M Potassium phosphate buffer (pH 6.5 containing 1.4 mM β-mercaptoethanol). The extract was centrifuged at 12,000g for 20min at 4°C. A 2ml reaction mixture (0.1ml CDNB (1-chloro-2,4-dinitrobenzene), 0.2ml of reduced glutathione (GSH) were added to 1.65ml of 10mM phosphate buffer (pH 6.8) and 0.05ml enzyme source was added. The increase in the absorbance value was recorded at intervals of 30s for 3 min.

Native PAGE (Davis 1964)

Native polyacrylamide gel electrophoresis (nPAGE) was performed by following the method given by Davis, 1964. The running and stacking gels of 7.5% and 5% polyacrylamide was used respectively for determining the number of bands corresponding to different enzymes and the gel was stained with commasie brilliant blue for visualization of band

Native PAGE of Esterases

10 insects each of the control and the insecticide treatment population of the white backed plant hopper were homogenized in 100-150µl 50mM phosphate buffer (pH 7.4)

containing 10mM Dithiothreitol (DTT) and centrifuged at 12,000g for 15 min and 4°C. 80µl of supernatant was mixed with 20µl of marker (bromophenol blue+ xylene cyanol in sucrose) and loaded onto a 7.5% polyacrylamide gel within Atto Electrophoresis Mini Gel System (Japan). Electrophoresis occurred in the electrode buffer (containing 100mM Tris, 2.4mM ethylene diamine tetraacetic acid (EDTA) and 100mM boric acid; pH 8.0). After electrophoresis, the gels were incubated in 100 ml of 20mM phosphate buffer (pH 6.8) containing 0.45mM 1-napthyl acetate (NA), and 0.45mM 2-NA. After 5 min, 25 mg Fast Blue BN salt (in 1 ml water) was added to visualize esterase bands. Approximately 5 min after adding Fast Blue BN, the gels were removed and fixed in 10% acetic acid.

RESULTS

LC_{50} determination

Table 2 shows LC_{50} values evaluated for each insecticide by using probit analysis. From the three insecticides tested buprofezin was found to be the most effective. Buprofezin was highly toxic with LC_{50} (0.001ppm) while chlorntraniliproleand quinalphos registered LC_{50} values of 2.17 and 1.64 ppm respectively.

concentration and about 70% insects in highest concentration. Similarly Fig. 2 and 3 shows percentage mortality of insects towards buprofezin and chlorntraniliprole.

Perusal of data in Table 3 reveal that the AchE activity in quinalphos treated insects was 4.6 fold than that in the buprofezin treated insects. AchE activity was lowest in chlorntraniliprole (3.2 µg of free thiol group formed/min/mg of insect). The highest activity of Esterases for the substrate p-nitrophenyl acetate was seen for quinalphos (30.82 µg of napthol formed/min/mg of insect) and the lowest activity was seen for chlorntraniliprole (14.40 µg of napthol formed/min/mg of insect). The highest GST activity was observed in chlorntraniliprole (2.88 µg/min/mg of insect) that was 5 fold as compared to control, however no or negligible increase in activity was observed in case of buprofezin and quinalphos as compared to control. Figure 4 shows that the highest AchE and esterase activity (µg/min/mg of insect) was observed in quinalphos and chlorntraniliprole shows highest GST activity.

Table 2. LC_{50}value ofthe fifth instar *Sogatellafurcifera* larvaeexposedto buprofezin, quinalphos and chlorntraniliprolafter 24 hobservation

Insecticide	Total No. of nymph used	LC_{50}value(in ppm)	Slope	Chi-square	df
Buprofezin	15	.001	.296+-.072	1.1013	6
Quinalphos	15	1.6484	.178+-.065	2.4804	6
Chlorntraniliprole	15	2.71698	.237+-.154	.3042	6

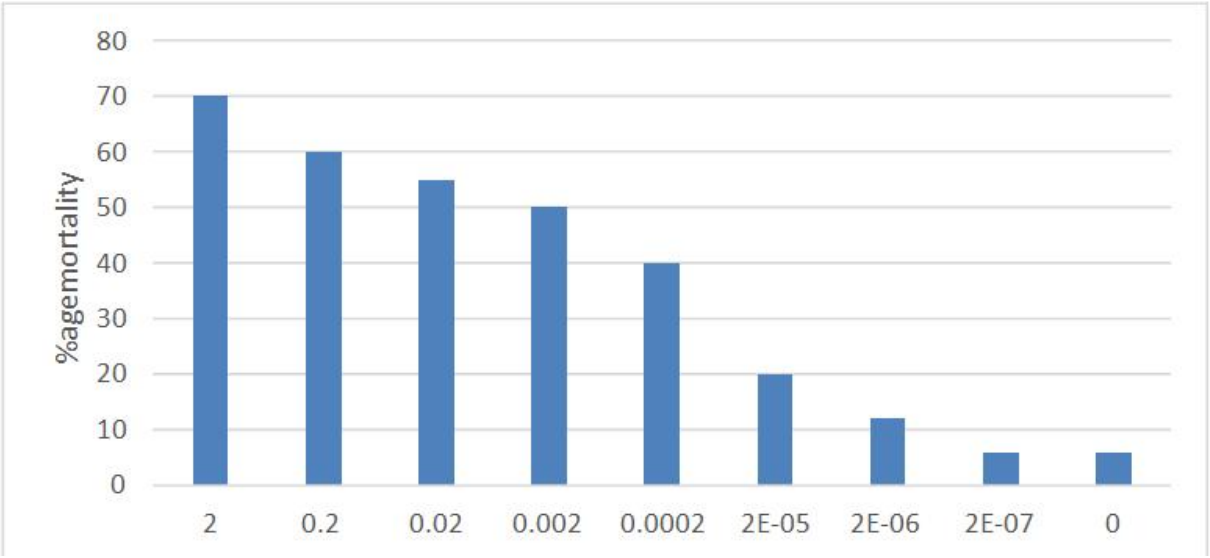

Figure 1. Dosage-mortalityresponses of WBPH toquinalphos

Figure 1 shows the mortality percentage of insects towards quinalphos after 72 hrs of treatment with eight insecticide dilutions. 0.002 ppm was corresponding to the 50 percent mortality.Lesser than 10% insects died in the lowest

Native PAGE (Fig. 5**)**

Native polyacrylamide gel electrophoresis revealed the presence of three bands which were designated as B1-B3 in

whole body homogenate of adult whitebacked planthopper, when 20µl of homogenate was loaded into each wells and commassie brilliant blue was used to stain bands.

Native PAGE of Esterase

Native PAGE when stained for esterase revealed the presence of one light band designated as E1 in whole body homogenate of WBPH without any insecticide application. (Fig. 6).

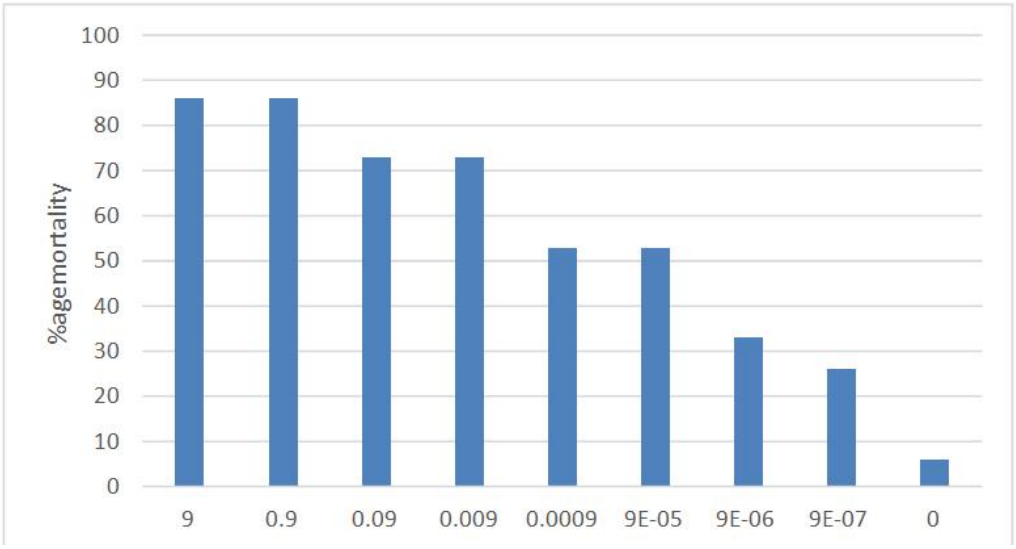

Figure 2. Dosage-mortalityresponses of WBPH tobuprofezin

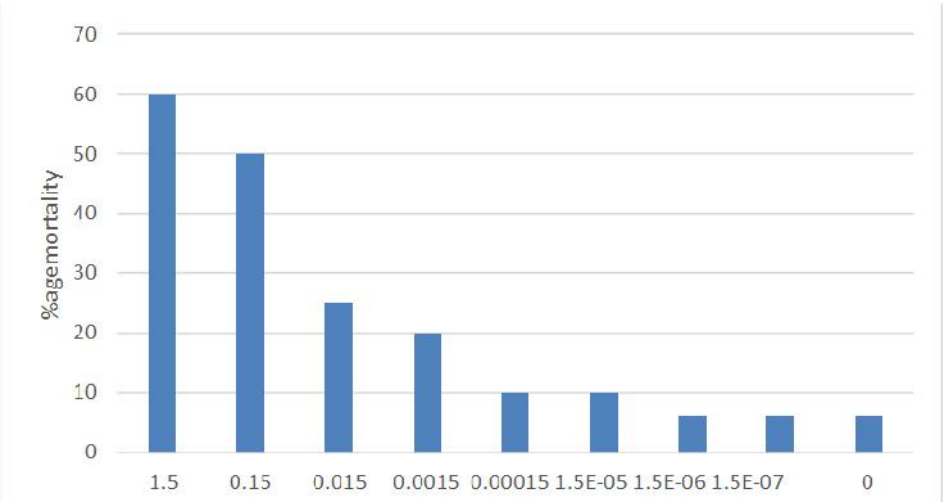

Figure 3. Dosage-mortalityresponses of WBPH tochlorntraniliprole

Table 3. Activityof AchE, Esterase and GSTenzymesin8thgenerationadult white backed planthopperin response to insecticides buprofezin, chlorantraniliprole quinalphos

Insecticide used	AchE (µg of freethiolgroup formed/min/mg of insect)	Esterase (µg of naptholformed/min /mgof insect)	GST(µg/min/mg of GSHconjugate formed of insect)
Buprofezin	3.60	29.65	0.59
Quinalphos	17.23	30.82	0.63
Chlorntraniliprol	3.22	14.40	2.88
Control	2.96	14	0.54

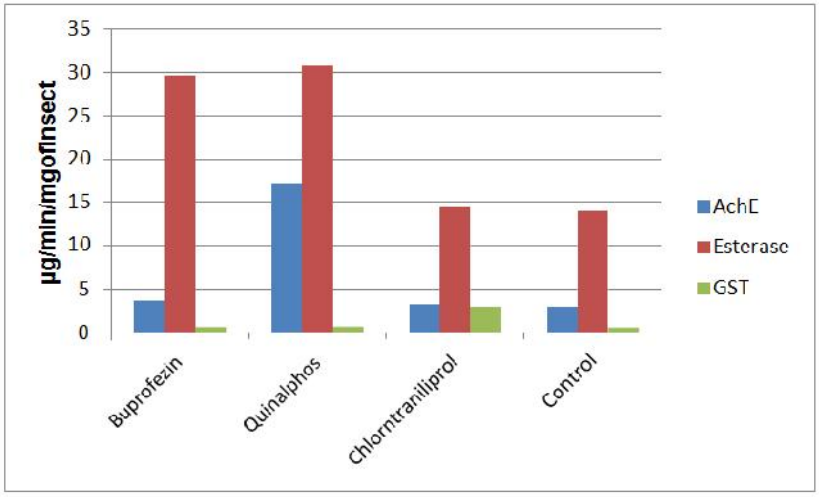

Figure 4. Detoxification enzyme profile to wards Buprofezin, Quinalphos and Chlorntraniliprolon 8th generation adult white back edplanthopper

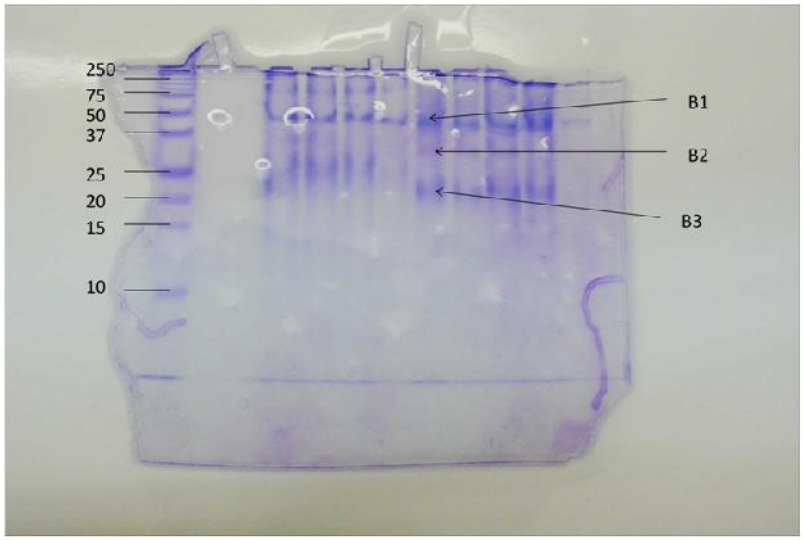

Figure 5. Nativepolyacrylamidegel (7.5% runninggel and 5% stackinggel) electrophoresisin Whole body homogenate of adult WBPH

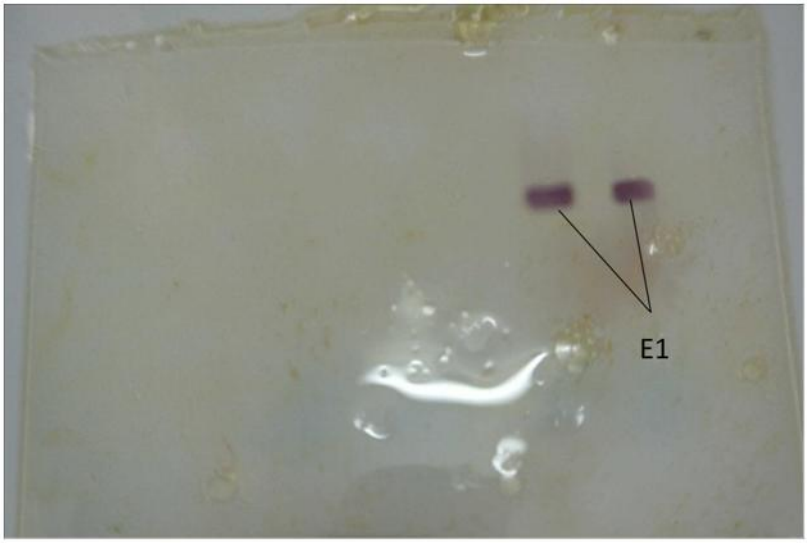

Figure 6. Native PAGE of esterases in whole body homogenate of adult WBPH (notsprayed with anyinsecticide)

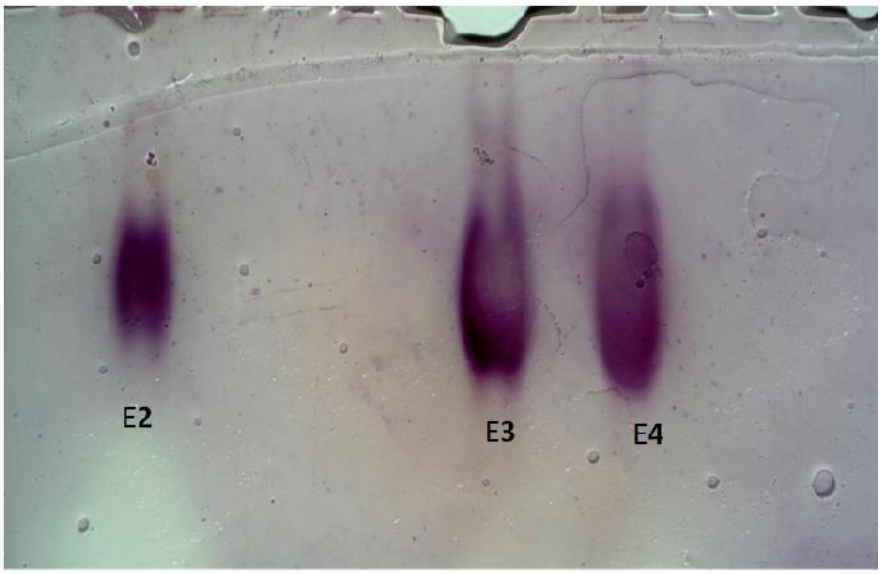

Figure 7. Native PAGE of esteraseextractedfrom8th generation population which was sprayed with buprofezin, quinal phosand chlorntraniliprol. Lane 1shows dark diffused bands E2, while lane2 and 3 shows E3and E4 respectively.

Esterase activity got enhanced on exposure to insecticide in WBPH as seen in Fig 7, which shows diffused dark bands in case of quinalphos resistant insects designated as E2, one dark purple colored band E3 was observed for buprofezin treated insects, however light colored diffused band E4 was observed in chlorntraniliprole selection pressure. Dark band in quinalphos resisted insects attributed for development of new esterase isoforms, while in case of chlorntraniliprole selection pressure, light colored bands show least esterase activity towards resistance mechanism.

DISCUSSION

Buprofezin, chlorntraniliprole and quinalphos have been widely used for control of *S. furcifera* and other insect pests of rice. A range of insect species have been reported to develop resistance. It has been studied that metabolic enzymes esterase and acetylcholinesterase are dominant organophosphates detoxification enzymes (Muthusamy *et al* 2013). These enzymes have significant contribution in xenobiotic detoxification in many rice insect pests (Karunaratne and Weerakoon, 2007). Detoxification enzymes can alter their forms to resist the insecticides. The activity of acetylcholinesterase increased which made the insect less sensitive towards the insecticide (Yang *et al* 2008). Furthermore both qualitative and quantitative modifications of AChE had evolved in the resistant strains and were likely to significantly enhance the overall resistance level in greenbugs (Zhu and Gao., 1999). An altered acetylcholinesterase (AChE) with poorer catalytic efficiency for the substrate acetylthiocholine iodide and 5- to 16-fold lower sensitivity to inhibition by omethoate was the major resistance mechanism (Vontas *et al* 2001). The results indicated that the insecticide resistance observed in the field strain was due to multiple resistance mechanisms, including increased detoxification of these insecticides by microsomal oxidases, glutathione S-transferases, hydrolases and reductases, and target site insensitivity such as insensitive acetylcholinesterase (Yu *et al.,* 2003).

The results of present study also showed increased acetylcholinesterase activity in quinalphos treatment (17.23 µg/min/mg of protein). This higher activity can be attributed to insensitivity of WBPH to quinalphos, an organophosphate insecticide. AchE insensitivity is known to be a dominant feature in resistant insects (Charpentier and Fournier, 2001).

Esterases have been known to be important detoxification enzymes in several insects like *Amsacta albistriga* (Muthusamy *et al.,* 2013) and *N. lugens* (Zhang *et al.*, 2014). The result of present study are in line that esterase activity was high in buprofezin and quinalphos selective strains as compared to the control.GSTs prevail in Cytosolic, mitochondrial and microsomal proteins. They catalyze the detoxification reactions in a number of ways. These detoxify the xenobiotic compounds by dehyrochlorination. During the current research, the activity of GST increased in the chlorntraniliprole resistant strains. To explain this effect, we hypothesize that the GSH got oxidized and the $H:^-$ ion participated in nucleophilic substitution with Cl^- ion of chlorntraniliprole to detoxify the insecticide. However biochemical mechanism of chlorntraniliprole is unknown. A high level of GST has been associated with insecticide detoxification in *N. lugens* (Karunaratne and Weerakoon, 2007). Vallas *et al.,* 2000 described a strong correlation in *B. germanica* between the increase of GST activity and exposure/resistance to several pesticide groups. In our experiment, it is clear that the increase in GST activity for chlorntraniliprole (2.88 µg/min/mg of fresh tissue) related to the insecticide resistance. Similar results were observed for chlorntraniliprole affected *H. armigera* (Cao *et al*., 2010).

Elevated esterase activity and expression of dark esterase band have been observed in resistance strain of *H. virescens* (Goh *et al*., 1995) and *Boophilus microplus* (Baffi *et al.,* 2005) in *Helicoverpa armigera* (Srinivas *et al.,* 2004). Significant correlations were found between resistance to insecticides and presence of group of three intensely stained bands (Lopez-

Soler *et al.,* 2008) The difference in staining intensity of esterase bands in response to dichlorvos treatment in *Amsacta albistriga* was examined (Muthusamy *et al.,* 2013). In our study we also find dark intense bands that might corresponds to some esterase isozymes developed to resist the insecticides.

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