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MOLECULAR CHARACTERIZATION OF RYNAXYPYR RESISTANT *PLUTELLA XYLOSTELLA* (L.)

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ABSTRACT
Present investigation was undertaken to know the biochemical and molecular mechanism of rynaxypyr resistance in Diamondback moth (P. *xylostella*.) Selection of DBM against rynaxypyr upto seven generations resulted in development of about 34.88 folds resistance. Biochemical studies revealed increased Glutathione-s-transferase and Carboxylesterase activity by 2.385 and 1.57 folds respectively, in resistant strain as compared to susceptible strain. Native PAGE demonstrated the induced and over expression of the respective enzymes. In RAPD-PCR out of 20 primers two primers gave maximum polymorphism and found to be potential markers for rynaxypyr resistance in DBM.

INTRODUCTION

Plutella xylostella commonly called as Diamondback moth is a serious pest of cruciferous crops like cabbage, cauliflower, knoll khol, radish etc. It is one of the most widely distributed insect in the world being reported from more than eight countries (Mohan and Gujar, 2003). The diamondback moth was first recorded in India in 1914 infesting cruciferous vegetables (Fletcher, 1914). The larva of this insect feeds on the foliage of cruciferous plant from the seedling stage to harvest and greatly reduce the quality and yield of produce. The first incidence of diamondback moth resistance in India was reported against DDT (Verma and Sandhu, 1968). DBM has shown highest resistance levels to synthetic pyrethroids (2814 to 46134 fold), followed by organophosphates (5 to 2726 fold) and carbamates (36 to 108 fold), while it was low for endosulfan (160-fold) and B. thuringiensis (2 to 15 fold) (Vastrad *et al.*, 2002)

Enzymes play an important role in detoxification of the insecticides in insect body. A family of multifunctional isozymes found in all eukaryotes, catalyses xenobiotics, including pesticides, resulting in elimination of toxic compounds (Dukre *et al.*, 2009). In insects Glutathione-S-transferase has major role for neutralizing or detoxifying the insecticides (Renuka *et al.*, 2003). Glutathione forms conjugate and eliminate the toxic compounds through and lead the mercapturic acid pathway and can be used as biochemical markers to detect chemical toxicity (Veenapani *et al.*, 2010). Esterases are associated with organophosphate resistance, by catalyzing t he hydrolysis of insecticides. Esterases play a significant role by undergoing metabolic degradation or by inactivating the toxicant by a physical or chemical process called sequestration (Devonshire and Field, 1991).

Rynaxypyr controls insect pest through a new mode of action. It activates Rynodine receptors (RyRs) Rynodine receptors are the distinct class of ligand gated calcium channels controlling the release of calcium from intracellular stores. Rynaxypyr, the anthranilic diamide, developed by Dupont Co. Ltd. act on this rynodine receptor (Settele *et al.*, 2008).

Diamondback moth has long history of eventually becoming resistant to every insecticide used extensively against, in many countries. In India there is no information available regarding or associated with mechanism of resistance to rynaxypyr molecule in *P. xylostella*. The research was undertaken to identify the development of resistance mechanisms in DBM against rynaxypyr. On that basis we can develop the strategies which could responsible for delaying the resistance in DBM against rynaxypyr. In the same time we could reduce the cost of cultivation of farmers

MATERIALS AND METHODS

Rearing of insect

The insects were reared on mustard seedlings, methods used to rear larvae and adults were essentially as described by Liu and Sun (1984). Continuous colonies of *Plutella xylostella* from different geographical locations were reared in the

laboratory under controlled conditions of temperature 25ºC ± 2ºC, 75 ± 5 per cent relative humidity and photoperiod of 13 hrs light: 11 hrs dark. Mustard seeds were soaked for 12-24 hrs in water and then sown in plastic cups with soil rite / peat moss and coir peat (20-25 seeds/cup). The seedlings were placed in mating chamber for oviposition once in two days. The adults were provided with liquid adult diet. After hatching, the neonate larvae mine into the mustard seedlings. Subsequently, the larvae were transferred to fresh seedling.

Selection procedure

Leaf dip method of bioassay as described by Tabashnik *et al.* (1987) was adopted in the present studies. Cabbage leaves were first washed with distilled water containing 0.1 per cent Triton X-100 and dried for about 1 hrs. Cabbage leaf disc (5 cm diameter) were cut and then dipped in a test solution for 10 seconds. The leaf disc was placed for about 2 minutes over a blotting paper in a tray to drain excess solution at room temperature. Ten third instar larvae (5 days old) were released on each disc in an individual petriplate where in blotting paper was placed at the bottom. Three replications were used for each concentration. The bioassay were conducted at room temperature. Similarly ten larvae were released on cabbage leaf disc dipped in water only, which was treated as control.

Preparation of enzyme

The third instar larvae (weighing 3.0 - 4.0 mg approximately were separated and starved for 7-8 hours to remove all digested food particles. Whole larvae were homogenized using mortar and pestle in sodium phosphate buffer (PB) (100 mM, pH 6.5), containing 0.1 mM of EDTA, PTU and PMSF each; insects were chilled in refrigerator before homogenization. The homogenate thus obtained was centrifuged at 10,000 rpm for 15 minutes at 4ºC in high speed refrigerated centrifuge, solid debris and cellular material was discarded. The resultant post mitochondrial supernatant obtained was stored at -20ºC and used as enzyme source. The protein was estimated by Bradford method (1976).

Glutathione s transferase quantification

GST quantification was carried out by method described by kao *et al* (1989). 50 μL of 50 mM1-chloro -2-4 dinitrobenzene (CDNB) and 150 μL of 5 mM reduced glutathione (GSH) were added in 2.77 mL of PB (100 mM, pH 6.5 with 0.1 mM PTU). After adding 30 μL of enzyme stock to the above mixture the content was shaked gently and incubated for 2-3 minutes, at 25ºC. The content was transferred into a 4mL cuvette and placed in the sample cuvette slot of the spectrophotometer. The absorbance for 5 min at 340 çm was recorded. The increase in absorbance over 5 minutes for calculation was taken into consideration. The samples were taken in triplicate. The enzyme activity was calculated as

$$\text{CDNB - GSH conjugate } \mu\text{M mg protein}^{-1}\text{ min}^{-1} = \frac{\text{Abs (increase in 5 min.) x 3 x 1000}}{\text{*9.6 x 5 x mg of protein}}$$

* 9.6 mM / cm - extinction coefficient for CDNB - GSH conjugate.

Carboxylesterase quantification

Carboxylesterase quantification was carried out by method described by Mohan and Gujar (2003). Substrate solution was freshly prepared with á-napthyl acetate (30 mM) dissolved in 1 ml acetone and volume made to 10 ml with SPB (0.04 M, 6.8 pH). Staining solution freshly prepared with 2 part of 1% Fast Brilliant Blue BB salt in SBP (0.04 M, 6.8 pH) and 5 part of 5% SDS in DD H_2O. 20 μL enzyme added to well of microplate with three replicates followed by 30 μL SPB (0.04 M, 6.8 pH). 100 μL substrate solutions were added to each well. Plate was incubated at room temperature for 30 min. After incubation, added 100 μL of staining solution to it in dark and plate kept in dark for 30 min. Blank also taken along with samples. After incubation absorbance was taken on the microplate reader. Calculations done by substracting blank from sample reading and resulted values were plotted on standard curve to get carboxylesterase concentration of samples.

Staining for GST activity on native PAGE

Native 10% PAGE was run at 4ºC for 4-5 hrs until the running front reaches the bottom of the gel. No SDS was added to any of the component of electrophoresis.

Staining solution-I : 100 mL 0.1 M sodium phosphate buffer, pH 6.5, containing 5.0 mM reduced glutathione and 1 mM each of CDNB (1- chloro -2, 4- dinitrobenzene) and Nitro Blue Tetrazolium (NBT)

Staining solution –II: 100 ml Tris HCL buffer, pH 9.6 containing 4 mM Phenozine methosulfate (PMS).

Staining for non specific esterases on native PAGE

After electrophoresis, the gel was placed in 100mL of 0.1 M sodium phosphate buffer (pH 6.5) containing 20mg of á napthol in 2mL of acetone and 100 mg of Fast Blue BB salt that was prepared and filtered just before use (substrate dye solution).

DNA extraction and Quantification

DNA was extracted using universal and rapid salt extraction method given by Salah and Martinez (1997). For total DNA isolation, Third instar larvae weighing about 50 to 100 mg

Table 1: List of RAPD primers used in the present study and their sequence

Sr. No.	Primer code	5' to 3' sequence
1)	OPD 01	ACC GCG AAG G
2)	OPD 02	GGA CCC ACC C
3)	OPD 03	GTC GCC GTC A
4)	OPD 04	TCT GGT GAG G
5)	OPD 05	TGA GCG GAC A
6)	OPD 06	ACC TGA ACG G
7)	OPD 07	TTG GCA CGG G
8)	OPD 08	GTG TGC CCC A
9)	OPD 09	CTC TGG AGA C
10)	OPD 10	GGT CTA CAC C
11)	OPD 11	AGC GCC ATT G
12)	OPD 12	CAC CGT ATC C
13)	OPD 13	GGG GTG ACG A
14)	OPD 14	CTT CCC CAA G
15)	OPD 15	CAT CCG TGC T
16)	OPD 16	AGG GCG TAA G
17)	OPD 17	TTT CCC ACG G
18)	OPD 18	GAG AGC CAA C
19)	OPD 19	CTG GGG ACT T
20)	OPD 20	ACC CGG TCA C

Table 2: Selection responses of *P. xylostella* to rynaxypyr over generations

Sr. No.	Selected generation	Probit analysis parameters						
		LC_{50} (ppm)	LC_{90} (ppm)	Fiducial limits of LC_{50}	Chi. Square	Slope	Regression equation	Resistance ratio
1)	F_5	0.0575	0.7244	0.0366 - 0.0904	6.4320	1.1647	Y = 6.445 + 1.1647X	-
2)	F_6	0.1553	1.1395	0.1035 - 0.2287	4.4306	1.4809	Y = 6.1979 + 1.4809X	2.700
3)	F_7	0.6772	1.3810	0.5789 - 0.7922	2.7839	4.1420	Y = 5.7011 + 4.1420X	11.77
4)	F_8	1.0270	1.6884	0.9346 - 1.1303	1.9754	5.9465	Y = 4.9292 + 5.9465X	17.86
5)	F_9	1.2310	2.4054	1.0809 - 1.4019	2.5066	4.4062	Y = 4.6023 + 4.4062X	21.40
6)	F_{10}	1.7382	2.973	1.5431 - 1.9580	2.7200	5.4965	Y = 3.6803 + 5.4965X	30.22
7)	F_{11}	2.0059	3.9492	1.7935 - 2.2434	0.6498	6.6639	Y = 2.9854 + 6.6639X	34.88

Table 3: Screening of rynaxypyr resistant and susceptible strain of DBM using RAPD marker

Sr. No.	Primer	Total number of bands	Number of polymorphic bands	Nature of the marker	Percent polymorphism
1)	OPD-1	4	0	Monomorphic	0.00%
2)	OPD-2	13	1	Polymorphic	7.69%
3)	OPD-3	13	1	Polymorphic	7.69%
4)	OPD-4	2	0	Monomorphic	0.00%
5)	OPD-5	10	0	Monomorphic	0.00%
6)	OPD-6	9	1	Polymorphic	11.11%
7)	OPD-7	1	0	Monomorphic	0.00%
8)	OPD-8	8	2	Polymorphic	25.00%
9)	OPD-9	2	0	Monomorphic	0.00%
10)	OPD-10	6	2	Polymorphic	33.33%
11)	OPD-11	7	1	Polymorphic	14.28%
12)	OPD-12	8	2	Polymorphic	25.00%
13)	OPD-13	5	1	Polymorphic	20.00%
14)	OPD-15	6	0	Monomorphic	0.00%
15)	OPD-16	7	1	Polymorphic	14.28%
16)	OPD-18	14	4	Polymorphic	28.57%
17)	OPD-20	10	2	Polymorphic	20.00%
	Total	125	18	-	14.40%

were thoroughly macerated with micro pestle in a 1.5 mL Eppendorf microcentrifuge tube containing 400 μL of sterile salt homogenizing buffer followed by 40 μL of 20% SDS and 8 μL of 20 mg /mL proteinase K and samples were incubated at 55 - 65ºC for overnight. Then 300 μL of 6 M Nacl was added to each sample. Samples were vortexed for 30 sec. at maximum speed and tubes spun down for 30 min. at 10000 g. The supernatant was then transferred to fresh tubes. An equal volume of isopropanol was added to each sample mixed well and centrifuged for 20 min., 4ºC at 10000 g. The pellet was washed with 70% ethanol dried and finally resuspended in 300-500μL sterile distilled water. Prior to RAPD characterization DNA quality assessed by agarose gel electrophoresis (0.7% prepared in TAE buffer) was of high molecular weight with DNA band near the wells and no streaking or RNA band. DNA concentration assessed at 260 nm in spectrophotometer.

PCR and RAPD analysis

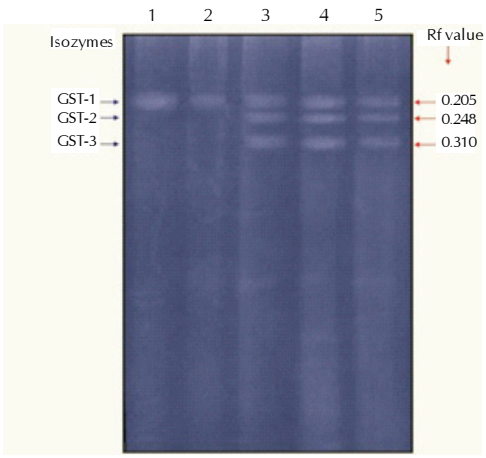
Twenty Operon primers (IDT) belonging to OPD series were initially screened and out of those, 17 primers showing good amplification with discrete fragments and polymorphism were selected for studying insecticide resistance. The PCR reaction was performed with a reaction volume of 25μL containing 1X PCR buffer ,1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2μM primer, 1U *Taq DNA polymerase* and 50 ng template DNA. Thermal Cycler (Eppedorf) programmed to fulfill 35 cycles after an initial denaturation cycle for 3 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 36ºC for 1 min and an elongation step at 72ºC for 2 min. The primer extension segment was extended to 5 min at 72ºC in the final cycle.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 μg/mL) in 1X TBE buffer at 80 volts for three hr. with a e DNA/Pst I marker, 24 (MBI Fermentas) along the samples. PCR products were visualized on UV light and photographed using Gel Documentation system.

RESULTS

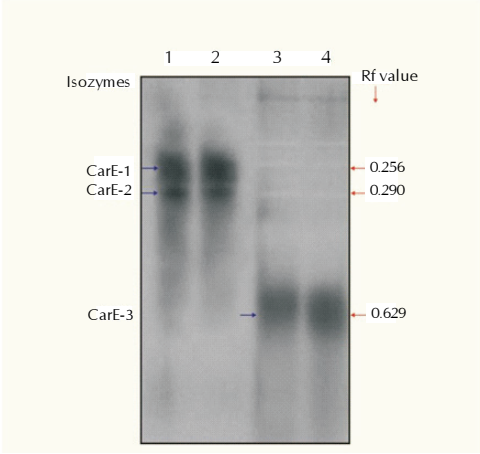
For determining LC_{50} value the homogeneous F_5 population was subjected to log dose probit (ldp) assay by leaf dip method of rynaxypyr against third instar larvae of *P. xylostella*. LC_{50} value of 0.057 ppm was observed for rynaxypyr against third instar *P. xylostella* larvae of F_5 generation.

Generation wise studies against rynaxypyr revealed that the resistance increased with increase in the number of selection regimes under insecticide pressure. The LC_{50} value of F_{11} selected population of *P. xylostella* against rynaxypyr was found to be 2.0059 ppm. It was 34.88 fold greater as compared



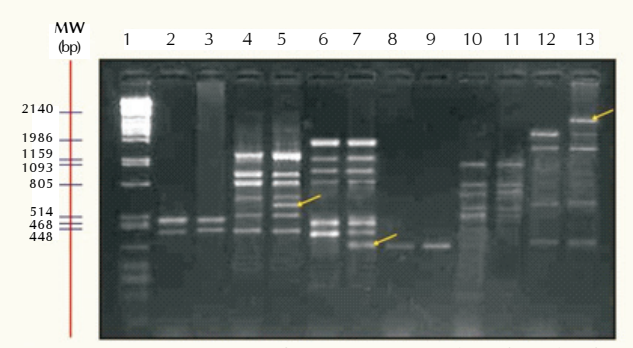
Note: Lane 1 and 2 - Susceptible strain, Lane 3, 4 and 5 - Resistant strain

Figure 1: GST isozyme pattern



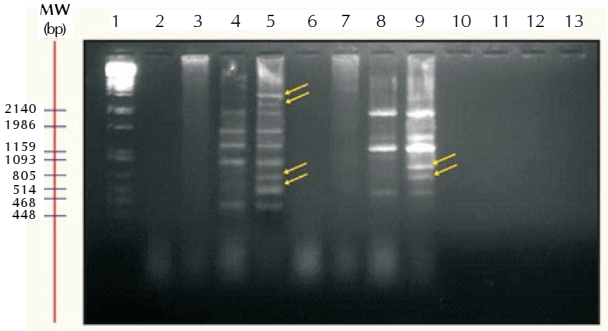
Note: Lane 1 and 2 - Resistant strain, Lane 3 and 4 - Susceptible strain

Figure 2: CarE isozyme pattern



Note: Lane 1 contains λ DNA/Pst I marker, (MBI Fermentas), after each set of two lanes results from amplification of template DNA obtained from susceptible and resistant DBM by OPD-11 (Lane 2-3), OPD 12 (Lane 4-5), OPD 13 (Lane 6-7), OPD 14 (Lane 10-11), OPD 16 (Lane 12-13)

Figure 3: RAPD marker (OPD 1-OPD-6) profile for Rynaxypyr resistant and susceptible Plutella xylostella



Note: Lane 1 contains λ DNA/Pst I marker, (MBI Fermentas), after that each set of two lanes results from amplification of template DNA obtained from susceptible and resistant DBM by OPD 7 (Lane 2-3), OPD 8 (Lane 4-5), OPD 9 (Lane 6-7), OPD 10 (Lane 8-9)

Figure 4: RAPD marker (OPD 7-OPD-10) profile for Rynaxypyr resistant and susceptible *Plutella xylostella*

to unselected F_5 population of *P. xylostella*.

Glutathione-S-transferase plays an important role in imparting resistance to insects. In present study the level of GST is varied in susceptible and resistant (selected) population. It was found that rynaxypyr selection pressure resulted in 2.385 fold increase in level of GST activity. In susceptible strain it was 1.762 μM mg protein^{-1} min^{-1} while in resistant strain it was found to be 4.202 μM mg protein^{-1} min^{-1}. This finding indicated the correlation between the level of GST and resistance to rynaxypyr.

It was observed that the level of carboxylesterase in crude homogenate of rynaxypyr resistant DBM strain was found to be 5.55 μM mg protein^{-1} min^{-1} which was 1.57 fold higher as compared to susceptible DBM strain which showed 3.57 μM mg protein^{-1} min^{-1} carboxylesterase activities. This increase in carboxylesterase activity in DBM-R strain as compared to DBM-S strain suggested the positive correlation between the enzyme activity and rynaxypyr resistance.

GST isozyme pattern

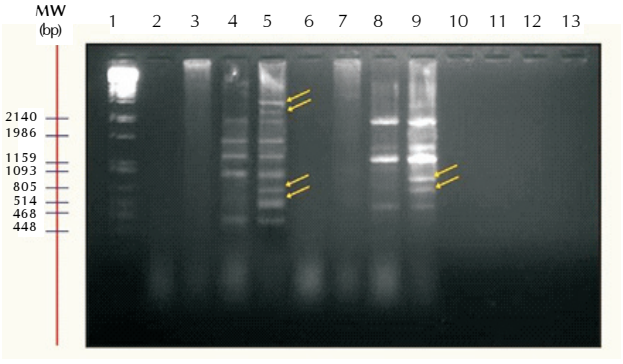
The native PAGE of homogenates prepared from whole body homogenates of rynaxypyr resistant and susceptible strains shows three GST isozymes having Rf values of 0.205, 0.248 and 0.310 denoted as GST-1, GST-2 and GST-3 respectively. According to mobility towards anode resistant and susceptible strains showed variation in banding pattern GST-1 with Rf value 0.205 was found in resistant as well as susceptible strain, while GST-2 and GST-3 were seen only in resistant strain.

CarE isozyme pattern

Study of esterase isozymes showed slow moving esterase isozymes in resistant strain with Rf value 0.256 and 0.290 while single fast moving esterase isozyme with Rf value 0.629 was seen in susceptible strain of DBM. Isozyme bands with varying intensities were observed, rynaxypyr resistant strain showed dark bands as compared to that in susceptible strain.

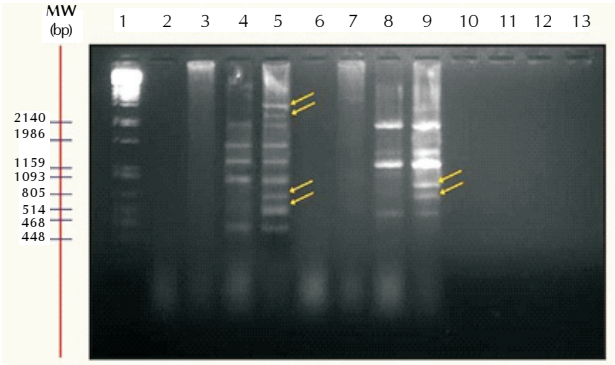
DNA polymorphism

Out of 20 primers screened during present study 3 primers did not give any amplification product. Six primers were monomorphic while 11 primers found polymorphic for the resistant and susceptible DBM population study. The 17 primers showing amplification generated total 125 bands out of which 18 bands were polymorphic and 107 bands were monomorphic.



Note: Lane 1 contains l DNA/Pst I marker, (MBI Fermentas), after that each set of two lanes results from amplification of template DNA obtained from susceptible and resistant DBM by OPD 11 (Lane 6-7), OPD 14 (Lane 8-9), OPD 15 (Lane 10-11), OPD 16 (Lane 12-13)

Figure 5: RAPD marker (OPD11-OPD16) profile for Rynaxypyr resistant and susceptible *Plutellaxylostella*



Note: Lane 1 contains λ DNA/Pst I marker, (MBI Fermentas), after that each set of two lanes results from amplification of template DNA obtained from susceptible and resistant DBM by OPD 17 (Lane 2-3), OPD 18 (Lane 4-5), OPD 19 (Lane 6-7), OPD 20 (Lane 8-9)

Figure 6: RAPD marker (OPD17-OPD 20) profile for Rynaxypyr resistant and susceptible *Plutella xylostella*

Primer OPD-10 showed maximum of 33.33 per cent polymorphism followed by primer OPD-18 with 28.57 per cent polymorphism, however other primers such as OPD-8 (25%), OPD-20 (20.00%), OPD-11 (14.28%), OPD-16 (14.28%), OPD-12 (25%), OPD-6 (11.11%), OPD-2 (7.69%) and OPD-3 (7.69%) showed polymorphism.

DISCUSSION

High resistance (about 34.88 folds) was developed in DBM against rynaxypyr in F_{11} generation, when DBM was screened against rynaxypyr in consecutive generation. Thus DBM has potential to develop resistance against rynaxypyr. Nirmal and Singh (2001) reported the development of resistance in DBM to the extent of 198-615 fold resistance to cypermethrin and 590 - 4576 fold resistance to fenvalerate. Cheng *et al.* (1997) reported 11 - 70 fold cypermethrin resistance to DBM in field condition.

As rynaxypyr is a newly introduced insecticide in the Indian farming system. The reference regarding resistance to this molecule is not available. From past study of some insects on different molecules it is showed that when the insect get exposed to the molecule the resistance increased by many folds when the selection pressure is applied, same phenomenon get reported in this study.

Increased level of GST (2.385 fold) and CarE (1.57 fold) detected in DBM resistant strain. This concluded that, these enzymes play significant role in rynaxypyr resistance in DBM. Similarly, Dukre (2009) reported 3.5 fold increase in GST activity in cypermithrin resistant DBM strain as compared to susceptible strain. In case of CarE, similar findings were obtained by Mohan and Gujar (2003). They found 1.2 to 1.8 fold increased carboxylesterase activity in pyrethroid resistant *Plutella xylostella*.

Native PAGE demonstrated the difference in GST as well as CarE isozymes when compared between resistant and susceptible strain. In case of GST, The isozyme pattern showed that the resistant strain might be showing induced expression of genes for detoxifying the rynaxypyr molecule while in case of CarE the gene products might be concentrated for detoxifying the rynaxypyr molecule. Hence this can be exploited as isozyme marker for distinguishing resistant and susceptible population of DBM against rynaxypyr. The role of higher activity of slow moving esterase identified by PAGE in insecticide resistance in *P. xylostella* was earlier reported by Maa and Liao (2000). Mohan and Gujar (2003) observed variation in the esterase isozyme profile of *P. xylostella* population collected from three locations in India and attributed to the differences in the use of insecticides in these locations.

RAPD markers are found to be an important molecular tool for discrimination between rynaxypyr resistant and susceptible strain of *Plutella xylostella* at DNA level. The amplicons that were either amplified or those that disappeared in the individuals surviving the effect of rynaxypyr selection pressure can serve as the potential RAPD markers for the identification of resistance at an early stage and could help in the pest management programmes. Early detection of resistance is helpful in the identification of effective insecticides to manage the pest. Significance of the fragments amplified only in the resistants is that these show the development of variants in the resistant DBM population after the selection pressure and hence can be used as possible markers to identify resistant individuals from a field population.

Sharma *et al.* (2008) studied the imidacloprid resistance in cotton whitefly using RAPD marker. The imidacloprid treatment produced distinct genetic alterations in surviving whitefly. Zhu *et al.* (1998) used RAPD-PCR technique for differentiating the deltamithrin resistant and susceptible *Culex pipens* to study the nucleotide divergence and insecticide resistance. Heckel *et al.* (1995) used RAPD technique for distinguishing *Bacillus thuringiensis* susceptible and resistant strains of DBM. Of 117 primers tested, 75 produced one or more bands that were found in one strain but absent in the other. More number of polymorphic bands were seen in resistant strain of DBM as compared to susceptible. Holeyachi and Savithramma (2013) used RAPD marker to screen the resistant genes associated with MYMV resistance in mungbean

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