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Identification of Rynaxypyr resistant *Plutella xylostella* with RAPD-PCR

KEYWORDS: RAPD PCR, Rynaxypyr, *P.xylostella*

Navid N Patel	Dr. K N Patel	Vina Sagane
Near Tamne Hospital,In Front of Devi temple, Kirti Nagar Akola.444004(M.S)	Near Tamne Hospital,In Front of Devi temple, Kirti Nagar Akola.444004(M.S)	Near Tamne Hospital,In Front of Devi temple, Kirti Nagar Akola.444004(M.S)

ABSTRACT *: RAPD PCR analysis was performed to know the molecular mechanism of rynaxypyr resistance in P. xylostella. Diamond backmoth(DBM) was selected against rynaxypyr upto seven generations. Selection process resulted in about 34.88 folds resistance to that of homogeneous susceptible F5 generation. RAPD-PCR analysis of rynaxypyr resistant and susceptible strain of P. xylostella showed considerable difference. Out of 20 primers screened 17 primers showed the amplification in which 11 primers found to be polymorphic in nature. Two primers gave maximum polymorphism and found potential markers for rynaxypyr resistance in DBM. Analysis of amplicons indicated that rynaxypyr treatment produced distinct genetic alterations in surviving P. xylostella.*

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).

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 these rynodine receptor (Settele et al., 2008).

Diamondback moth has long history of eventually becoming resistant to every insecticide like cypermethin fenvalerate, organophosphates, pyrethroids and spinosad used extensively against, in many countries Nirmal and Singh 2001, Joia et al., 1996, Mehrotra and Phokela 2000, Renuka and Regupathy 1996, Zhao 2002). In India there is no information available regarding or associated with mechanism of resistance to rynaxypyr molecule in P. xylostella, hence this study was undertaken.

MATERIAL 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 25°C ± 2°C, 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.

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 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 55-65°C 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., 4°C at 10000 g. The pellet was washed with 70% ethanol dried and finally resuspended in 300-500 µl 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 25 µl 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 94°C. Each cycle

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consisted of a denaturation step at 94^{0}C for 1 min, an annealing step at 36^{0}C for 1 min , and an elongation step at 72^{0}C for 2 min. The primer extension segment was extended to 5 min at 72^{0}C in the final cycle.

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

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 λ 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.

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

		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

Generationwise 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 to unselected F_5 population of P. xylostella,

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.

OPD 1- OPD 6
OPD11-OPD16

OPD 7-OPD-10
OPD17-OPD20

Table3. 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%

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:

The primers OPD-10 and OPD-18 showed higher polymorphism as compared to the other primers used in the present study indicated the usefulness of these primers in distinguishing the susceptibility and resistance of Plutella xylostella against rynaxypyr.

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 distinguish-

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ing 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.

In the present study 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 marker for the identification of resistance at an early stage and could help in pest management programmes.

The fragments amplified only in the survivors i.e. resistant showed the development of variants in the resistant population after the selection pressure and hence could be used to identify rynaxypyr resistant individuals from field population.

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