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Assessment of variation in isoproturon in susceptible and resistant biotypes of *Phalaris minor* Retz. by RAPD analysis

Rupa S Dhawan*, A K Dhawan, S Kajla and R Moudgil
CCS Haryana Agricultural University, Regional Research Station, Uchani, Karnal 132 001, India

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The extensive use of isoproturon for the control of weeds in wheat fields for the past 20 years has lead to the formation of isoproturon resistant biotypes of *Phalaris minor*. This problem was first identified in Haryana and later in Panjab and some parts of Uttar Pradesh. PCR based RAPD technique, which can detect variability at DNA level was used to assess the variability among the two isoproturon susceptible and two resistant biotypes of *Phalaris minor*. Nine oligonucleotides (10 base) were screened for their ability to produce polymorphic bands. Three primers did not amplify DNA of one or the other biotypes. Three more did not show any polymorphism amongst the biotypes. The rest three indicated 20% polymorphism level. The size of amplified DNA segments ranged from 105 to 1020 base pair (bp). The mean dissimilarity value of these biotypes was 0.19. Highest dissimilarity (0.21) could be observed between the two resistant biotypes. Cluster analysis of the RAPDs generated separated one of the resistant biotypes from rest of the populations. Primer 20 A0 showed maximum polymorphic value of 1.0 between the susceptible biotype from Karnal and the resistant biotype from Kalwehri. This suggests that the technique could be utilized in the assessment of genetic diversity of the populations existing in other states and developing markers for the resistant trait.

Keywords: *Phalaris minor*, isoproturon resistance, RAPD profiles

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Introduction

Phalaris minor Retz. is a self-pollinating weed infesting rabi crops, especially wheat in almost all wheat growing states of India. Isoproturon [N' (4-isopropyl-phenyl) N-N'dimethylurea], a urea herbicide has been in use to control this weed by affecting the photosynthetic system and thereby killing it[1]. Some of the populations of the weed have been observed to show resistance to this herbicide after its continuous use for over 10 years in Haryana, Panjab and Uttar Pradesh and resulted in drastic reduction in wheat yields[2-4]. The resistant biotypes have been observed to possess an increased activity of the enzyme cytochrome P450 monoxygenase leading to the detoxification of the herbicide[5,6]. A similar mechanism is operative in wheat[7].

There is not much apparent variability in the morphological characteristics of the susceptible and resistant biotypes. Some variations in physiological behaviour like increased germination and faster rate of seedling growth have been observed[8]. Increased activity of cytochrome P450 monoxygenase is suggestive of a change at genetic level. A need to develop biochemical and molecular markers was felt to differentiate susceptible and resistant biotypes. A comparative study of the protein profiles of isoproturon susceptible and resistant biotypes revealed variability amongst the susceptible biotypes and differences from the resistant biotypes, however, these could not be correlated with the resistant trait[9]. A study of their RAPD profiles was planned to indicate the extent of variability in these biotypes and observe correlations with the resistant trait.

Materials and Methods

The seeds of susceptible biotypes were collected from CCS Haryana Agricultural University, Regional Research Station Karnal (S1), and from farms of HAU, Hisar (S2), from areas where there was no history of isoproturon spray. Those of the resistant biotypes were collected from villages surrounding Karnal viz. Uchana (R1) and Kalwehri (R2) where isoproturon resistance problem was seen. These had GR_{50} (50% growth reduction) values of 0.40 for S1, 0.30 for S2 and 1.7 and 2.25 for R1and R2. The seeds were sown in earthen pots in the screen house in

*Author for correspondence:
Tel: 91-184-221857
E-mail: rupadhawan@hotmail.com

November. After 2 months of growth, 100 mg of the tissue in two replicates was taken for RAPD analysis. It was washed thoroughly with mild detergent, and then with water. It was then rinsed in 70% alcohol, followed by 3-4 washings with double distilled water to remove the traces of alcohol. Leaf DNA was extracted using the kit developed by Bangalore Genie and stored at –20°C until used. The precipitated DNA was finally solubilized in 20 uL of solution B in the kit.

The DNA concentration was estimated by measuring absorbance at 260 nm with a UV spectrophotometer and samples diluted to obtain a concentration of 50 ng/ul. It generally required a dilution of 100 times. Nine decamer oligonucleotide primers were screened for their ability to produce repeatable polymorphic DNA bands using RAPD analysis. The details of the primers used are given in Table 1. The quality of DNA was tested by horizontal agarose (1.4%) gel electrophoresis. For RAPD analysis, the protocol described by Williams *et al*[10] for PCR amplification and agarose electrophoresis was employed with slight modifications.

PCR mix was prepared for RAPD reaction mixture in a volume of 25 uL in an Eppendorf tube containing 0.5 uL Taq polymerase (15 unit), 2.5 uL of 10 X assay buffer with 15 m*M* $MgCl_2$, 1.0 uL mix dNTP's-deoxynucleotide triphosphates (2.0 m*M* each); 1.0 uL primer (50-60 ng/mL) and 1.0 uL sample DNA (50 ng/uL). The mixture was spinned for 5 sec and kept in the thermocycler for amplification of the DNA. Amplification consisted of an initial denaturation for 6 min at 94°C. After this, 40 cycles of denaturation for 1 min at 94°C, annealing at 42°C for 2 min and extension for 1 min at 72°C was performed. After the final cycle, the samples were incubated for a further 5 min at 72°C and then kept at 4°C. One reagent control that included all the components of the PCR reaction except sample DNA was made for each amplification to monitor any possible contamination. Samples were loaded in the 1.4% agarose gel containing ethidium bromide (0.5 mg/mL). Electrophoresis was carried out in Tris-EDTA buffer. A 100 base pair ladder was also run in the gel along with the samples. PCR products were viewed under UV light using photodyne UV illuminator. Data generated from the sdetection of polymorphic segments were analyzed by employing the following equation of Nei and Li [11].

Similarity (F) = 2 Mx/My+ Mz

Dissimilarity = (1-F)

Mx= Number of shared segments between y and z

My = Number of scored segments of Y

Mz= Number of scored segments of Z

Cluster analysis on the mean dissimilarity values (1-F) between pairs of biotypes based on RAPDs generated was performed to calculate the genetic distances between populations.

Results and Discussion

There was not much apparent morphological dissimilarity in the susceptible and resistant biotypes. The rate of growth of the resistant biotypes was, however, observed to be higher in the initial stages as compared to the susceptible ones indicating better competitive ability of these biotypes (Table 1). The fact that morphological variability may not accompany genetic changes has been emphasized earlier[12,13].

Out of the 9 primers tested, 3 primers did not amplify DNA of one or the other biotypes. This may be because these primers may not have found their matching complements. The rest of the six primers

Table 1—Relative growth rates and RAPD data of isoproturon susceptible and resistant biotypes of *P. minor* Retz.

Biotype	Rate of germination 0-5 days after imbibition	Relative growth rate (mg/plant/day) 15-45 days after sowing	GR_{50} (Kg/ha)	RAPD data	
				Total bands	Mean dissimilarity value
Karnal Susceptible (S1)	7.8	0.04	0.40	33	0.11
Hisar Susceptible (S2)	3.8	0.07	0.30	37	0.07
Uchana Resistant (R1)	16.4	0.06	1.7	36	0.11
Kalwehri Resistant (R2)	16.2	0.08	2.25	31	0.14

produced a total of 41 repeatable bands, 8 of which were polymorphic and 33 monomorphic both among the susceptible and resistant populations (Table 2). This showed a polymorphic value of around 20%. Although *P. minor* is mainly an autogamous species the presence of polymorphism suggests that there may be a certain degree of cross-pollination within the species. Mean polymorphic value between different primers ranged from 0 for primer 40A10, 70B08 and 80B10 and to 0.75 for primer 20A0. There was no sequence relatedness amongst the primers that generated similar values. The amplified products generated by different primers are illustrated in Fig. 1. The size of amplified DNA fragments ranged from 150 to 1020 bp. Most of the amplified segments ranged in size between 300 and 900 bp. Number of fragments amplified by different primers was 33 in S1, 38 in S2, 36 in R1 and 32 in R2. Primer 20A0 generated maximum number of bands (10) while 80B10 generated a minimum number of bands (5). Primer 20A0 showed maximum polymorphic value of 1.0 between S1 and R2. This may be used as a marker for further studies to identify resistant populations at other locations. Polymorphic or the dissimilarity value (1-F) obtained from the pairwise comparison of RAPD bands amongst the 4 biotypes is shown in Table 3. The mean dissimilarity value of these biotypes was 0.109. Highest dissimilarity value was obtained between R1 and R2 (0.21). This was followed by dissimilarity between S1 and R2, which was 0.16 and between S1 and R1 to the level of 0.13. The dissimilarity observed between the two susceptible biotypes was 0.09. This is indicative of the fact that the susceptible populations at two locations show some genetic variability. The two resistant populations though located around Karnal also showed variability. Cluster analysis of the 1-F values group the two susceptible biotypes together with the resistant biotype from Uchana. The resistant

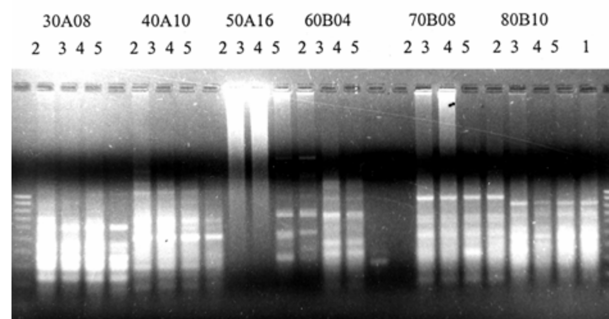

Fig. 1—RAPD amplification products against different random primers separated on 1.4% agarose and stained in ethidium bromide: 1= 1000-100 bp ladder; 2= susceptible biotype from Karnal (S1); 3= resistant biotype from Uchana (R1); 4= susceptible biotype from Hisar (S2); 5= resistant biotype from Kalwehri (R2)

Table 3—Genetic distance $(1\text{-F})^2$ values among pairs of *P. minor* biotypes based on RAPD's generated by six random sequence primers

Biotype	Karnal (S1)	Hisar (S2)	Uchana (R1)	Kalwehri (R2)
Karnal (S1)	0			
Hisar (S2)	0.09	0		
Uchana(R1)	0.13	0.03	0	
Kalwehri(R2)	0.16	0.14	0.21	0

Table 2—Number, name and sequence of primers used in PCR amplification of DNA, the number of amplified bands generated by different primers and mean polymorphic value in different *P. minor* biotypes

Oligo number	Name	Sequence(5'-3')	Bands scored			Mean (1-F)
			Poly morphic	Mono morphic	Total	
1100 40	Primer 20A0	GAAACGGGTG	3	7	10	0.75
1100 41	Primer 30A08	GTGATCGCAG	1	5	6	0.025
1100 42	Primer 40A10	TCGGCGATAG	0	7	7	0
1100 45	Primer 70B08	GTCCACACGC	0	7	7	0
1100 46	Primer 80B10	CTGCTGGGAC	0	5	5	0
1100 47	Primer 90B11	GTAGACCCGT	4	2	6	0.15
Total			8	33	41	

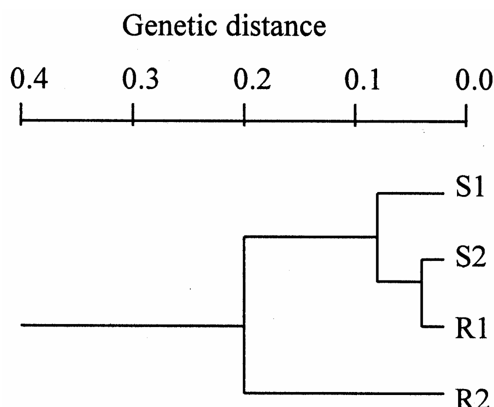

Fig. 2—Dendrogram for *P. minor* populations generated by cluster analysis of genetic distance values from random amplified polymorphic DNA data. Relative branch lengths indicate relative genetic distances between biotypes.

biotype from Kalwehri falls in a separate group (Fig. 2). The average inter cluster distance was 0.17. The intra cluster distance was 0.05. The study indicates that RAPD analysis is useful in assessing genetic diversity amongst *P. minor* biotypes. The grouping of resistant biotype from Uchana in the first cluster is indicative of the fact that although it has developed resistance to the herbicide isoproturon, it is still sufficiently similar to the susceptible biotypes genetically. A further study of other susceptible and resistant biotypes from other locations in India could lead to a better assessment of the genetic diversity within the species. The technique has been amply exploited in grouping herbicide resistant biotypes of *Echinocloa crusgalli*, a weed of rice fields. Independent modes of origin of resistant biotypes in different clusters have been observed[14]. The technique has also been exploited in monitoring spread of atrazine resistant *Solanum nigrum* in Europe[15].

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