HELIA, **28**, Nr. 43, p.p. 33-42, (2005) UDC UDC 633.854.78:631.523.632.1

ASSESSMENT OF HERITABILITY OF *Alternaria helianthi* RESISTANCE TRAIT IN SUNFLOWER USING MOLECULAR MARKERS

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Received: July 10, 2004
Accepted: November 22, 2005

SUMMARY

Leaf spot disease caused by *Alternaria helianthi* (Hansf.) is an important fungal disease of sunflower in India and elsewhere. Non-availability of known sources for resistance to Alternaria is a major constraint in sunflower breeding. Hence, seeds of sunflower genotypes were subjected to gamma-irradiation to develop mutant populations. Mutants were selected from the M_2 generation based on percent disease incidence and were passed on to the M_3 generation. Genomic DNA from leaf samples was evaluated by PCR amplification using random decamer primers and the amplified DNA fragments from mutants were compared with their respective controls. Single marker and stepwise regression analysis carried out in relation to percent disease index indicate that the alleles OPC5-B, K, J, OPA12-D and OPA15-A are strongly associated with *Alternaria helianthi* resistance.

Key words: sunflower, *Alternaria helianthi*, radiation-induced mutation, RAPD, plant disease index

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is an important oilseed crop which is presently grown at about 2.2 million ha in India. Alternaria blight, caused by the fungus *Alternaria helianthi* (Hansf.) Tubaki and Nishihara, is a serious foliar disease of sunflower. The disease is particularly destructive in tropical and sub-tropical regions wherein a combination of high temperature and extended periods of humid weather cause rapid epidemics (Sujatha *et al.*, 1997; Dudienas *et al.*, 1998). Under these conditions, the pathogen causes premature senescence and rapid defoliation

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(Kong *et al*., 1997). Seed yield is reduced up to 70% together with significant reduction in quantity and quality of oil (Kolte, 1990). Disease symptoms appear more frequently on older leaves than on young and expanding ones. Plants are highly susceptible during anthesis and foliage reduction at this stage results in 80% loss in yield (Allen *et al*., 1983; Carson, 1985; Kolte, 1990). Although utilization of genetic resistance is the most economical means of plant disease control, non-availability of resistance sources to Alternaria leaf spot disease has been major constraint in sunflower breeding programs. However, a few sources of resistance to *A. helianthi* have been identified and quantitative differences among genotypes have been reported; the usefulness of such levels of resistance under epidemic conditions seems to be limited (Morris *et al*., 1983; Carson, 1985; Lipps and Herr, 1986; Das *et al*., 1998). Almost all the genotypes and hybrids under cultivation are susceptible to leaf spot disease caused by *Alternaria helianthi* (Chattopadhyay, 1999).

Induced mutations have been subject of molecular investigation for the analysis of structure and function of related genes and their role in biotic and abiotic stresses. Further, the use of physical and chemical mutagens for developing useful mutants for crop improvement in the past has increased biodiversity and productivity in different parts of the world (Liu, 1990; Pathirana, 1992; Singh and Singh, 1996; Bhagwat and Duncan, 1998; Yoshika *et al*., 1999). In the present study, gamma-radiation was used to develop sunflower mutants resistant to *Alternaria helianthi*. Marker-assisted selection using RAPD markers is becoming a potential tool in crop improvement, for tagging pest and disease resistance genes (Lyngdoh, 2003; Lyngdoh, 2004; Melchinger, 1990; Raghavendra and Prasad, 2002; Uma *et al*., 2004; Welsh and McClelland, 1991), enabling more efficient identification and selection of genotypes carrying specific combinations of resistance genes. The present investigation also reports the use of RAPD profiles to analyze the mutants for stability of percent disease index (PDI) and also to identify markers with the leaf spot disease resistance in sunflower.

MATERIAL AND METHODS

Plant material

Seeds of five *cms* lines, their maintainers (A and B lines of 338, 343, 234, 336 and 17) and their restorers (RC-1, R 265, R 274, R 855 and R6D-1) were subjected to gamma irradiation (5, 10, 15, 20 and 25 kR) at Bhabha Atomic Research Center, Mumbai. The irradiated and control seeds were grown for three generations during the *kharif* season following standard package of practices for sunflower cultivation. Mutant lines of "A" were crossed with their respective "B" lines, while the "B" lines and restorers lines were selfed. Plant disease reactions were assessed using an *in vitro* toxin assay protocol (Prasad, 2003). Disease incidence in the field was

assessed by the appearance of leaf spot symptoms and was scored by using a visual rating system based on the coverage of the spots on the leaf:

0 - no symptoms on the leaves;

1 - <1% of the total leaf area affected;

2 - 1-5% of total leaf area affected;

3 - 25% of total leaf area affected;

4 - 25-50% of total leaf area affected and

5 - >5% of total leaf area affected). PDI was calculated using the following formula (Lyngdoh, 2003):

PDI = Sum of numerical rating / Number of leaves examined × 100 / Maximum disease grade (5)

RAPD analysis

DNA was extracted from sunflower leaves using CTAB method (Lyngdoh, 2003; Lyngdoh, 2004; Porebski *et al*., 1997; Uma *et al*., 2004) and 5 ng/l of DNA was used as template for PCR analysis. Amplification was achieved in a MJ research thermocycler (PTC 100) programmed for initial denaturation of 95°C for 4 min., followed by 40 cycles with denaturation at 94°C for 1 min., primer extension at 72°C for 2 min. and final extension was performed for 10 min. at 72°C. Amplified DNA fragments were resolved on 1.4% agarose gel electrophoresis containing 0.5 g/ml ethidium bromide. The gels were visualized under UV light and documented using Heto Laboratory gel documentation facility. RAPD profiles were generated for the mutant individuals and the control.

Statistical analysis

Bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The dissimilarity matrix was developed using squared Euclidean distance (SED), which estimated the pairwise differences in the amplification product (Sokal and Sneath, 1973). Dendrogram was computed based on Ward's method of clustering, using the minimum variance algorithm (Ward, 1963) from a statistical software package. Single-marker analysis by Fisher's method of analysis of variance was carried out to establish the association of markers with PDI. Regression (R^2) values were also determined to assess the amount of variability by considering PDI as dependent and molecular markers as independent variables. Stepwise regression analysis was carried out to identify the best combination of markers associated with PDI.

RESULTS AND DISCUSSION

The plant material used for this investigation was raised for three generations during the *kharif* season, when conditions are suitable for leaf spot infection (Dudi-

enas *et al.*, 1998; Das *et al.*, 1998). Screening the mutants resistant to leaf spot incidence was undertaken from the M_2 generation onwards. The mutants that showed lower PDI (%) as compared with the other genotypes/mutants/controls were forwarded to next generation for evaluation. Based on *in vitro* and field evaluations of PDI, 20 mutants belonging to 17B, 343B and 234B genotypes, which showed resistance to *Alternaria helianthi*, were identified.

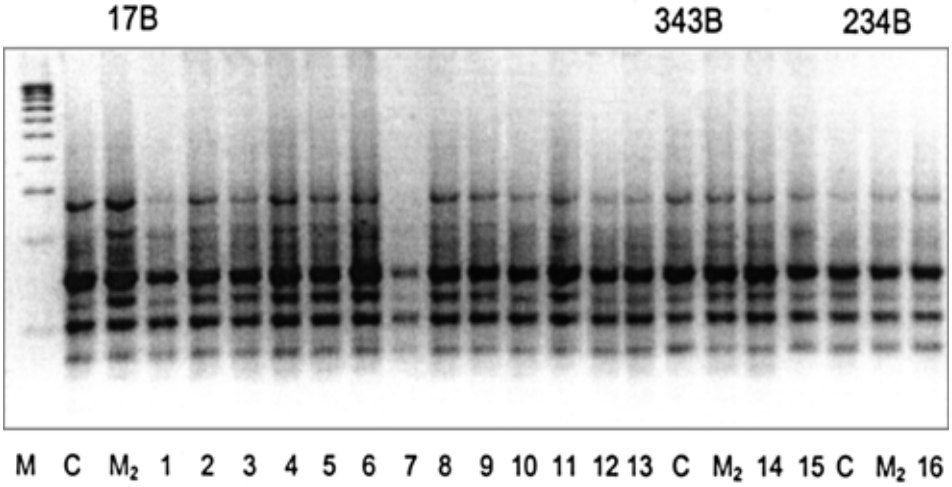

Figure 1: RAPD gel profile of the three genotypes of sunflower along with their mutants with primer OPA 12. M-DNA ladder marker - 500-5000 bp; lanes M_2 – mutants of respective sunflower genotypes at M_2 generation; lanes 1-13 – mutants at M_3 generations of 17B; lanes 14-15 mutants at M_3 generation of 343B; lanes 16 – mutant at M_3 generation of 234B.

Table 1: Percent disease index* for sunflower genotypes and their mutants

Genotype	Control		M_2 generation		M_3 generation	
	Range	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM
17B	41.0-50.4	44.9 ± 1.6	17.9-21.5	19.5 ± 0.6	17.6-21.8	19.7 ± 1.0
343B	44.4-48.7	45.9 ± 0.7	18.8-24.6	21.3 ± 0.8	19.4-22.0	20.7 ± 1.4
234B	42.5-47.8	45.2 ± 0.8	18.8-21.6	20.1 ± 0.6	20.7	-

Control: genotypes are non-irradiated and grown in similar conditions as their respective mutants
M_2 and M_3 represent the respective mutants that are proven resistant to *Alternaria helianthi* under field evaluation.
* PDI was calculated as described in the text

Among the 200 random decamer primers (Operon Technologies Inc., USA) screened, 10 primers that generated consistent, unambiguous and repeatable banding patterns were used to generate RAPD profiles of the parent genotypes, which ranged from 0.25 kb to 1.25 kb. Number of polymorphic bands specific to the mutants either by the presence or absence could be seen from the RAPD gel profiles generated by the selected primers. Of the 120 bands, 47.5% were polymorphic,

51.6% were monomorphic and 0.83% was unique only to one genotype. Primers OPA16, OPA17 and OPA19 produced the maximum number of polymorphic bands (14 bands each) followed by OPA14 and OPC4 (13 bands each) and OPB5, OPC5 (12 bands each).

The dissimilarity matrix was calculated using SED and dendrogram was constructed using Ward's method of clustering. The 21 individuals (including 15 mutant individuals of the M_3 generation and their respective M_2 and controls representing the three genotypes) clustered together at a distance of 100 units on the dendrogram with 17B-C and 17B M3-9, 10 spanning the extremes. All individuals clustered into 2 major groups of about 75 and 42 units. The first cluster consists of three mutant individuals, two of them belonging to the M_3 generation of 17B genotype and the other belonging to the M_2 generation of 234B. The second cluster being the largest, consists of all the three control and 15 mutant individuals belonging to M_2 and M_3 generations of the three genotypes chosen for investigation (Figure 2).

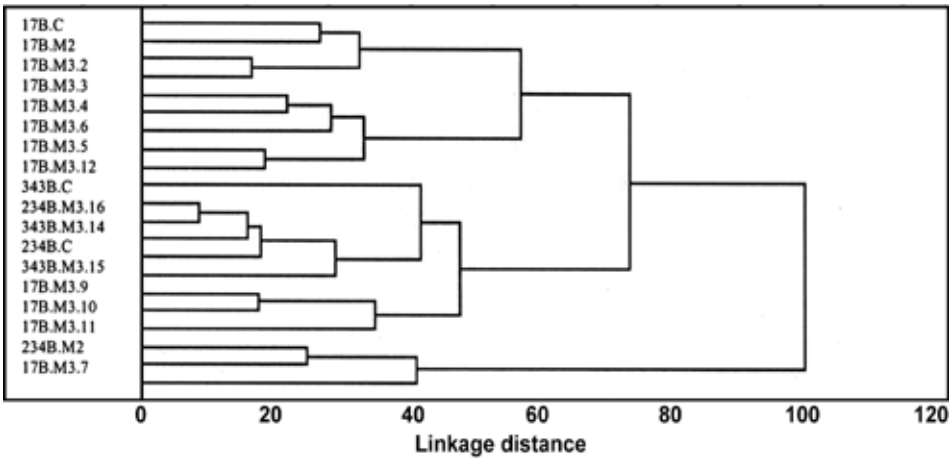

Figure 2: Polymorphism in sunflower genotypes and their mutants based on RAPD profiles generated from 10 primers with 23 individuals. C – control genotypes 17B, 343B and 234B, M_2: mutants of 17B, 343B and 234B and M_3: mutants 17B (13 No), 343B (2 No) and 234B (1 No).

The 14 individuals of 17B genotype clustered at a distance of 80 units with the 17B-control and M_3-9, 10 spanning the extremes and all other individuals clustering into two major groups at about 60 and 50 units (Figure 3). The genetic dissimilarity values between the control and M_3 individuals ranged from 30 to 45%, between M_2 and M_3 individuals from 19 to 46%, and among M_3 individuals from 17 to 63%. The 4 individuals of 343B genotype clustered together at a distance of 34 units with the control and M_3-06 spanning the extremes (data not shown). The largest group consisted of 343B-M_2, M_3-05 and M_3-06. The genetic dissimilarity values between control and M_3 varied between 29-33% and 14-24% between M_2 and M_3. M_3 individuals varied by ~26%. The 234B individuals clustered together at a dis-

tance of 49 units with the 234B-control and M_2 spanning the extremes (data not shown). The genetic dissimilarity between control and M_3 individual was 13% and 38% between the M_2 and M_3 individuals.

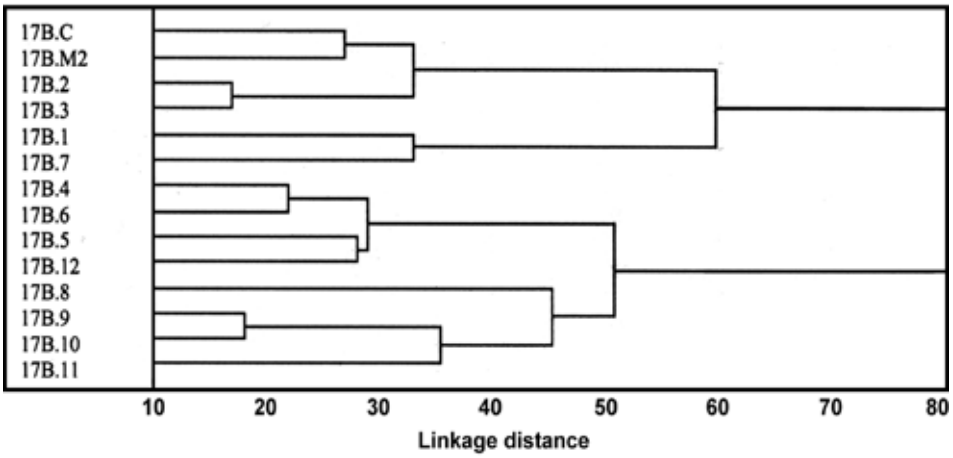

Figure 3: Dendrogram of the 17B genotypes (control) and their mutants (M_2 and M_3) of sunflower. The PDI was used as dependent and RAPD data were generated using the 10 primers as independent variables.

The clustering analysis for each of the three genotypes showed that the M_2 and M_3 individuals clustered together and far from the control except for 234B. The genetic dissimilarity values between the M_3 and M_2 individuals were found to be lower when compared with their respective controls. Examining the gel profiles, the pattern of bands generated by M_3 mutant individuals were found to be almost similar to that of M_2 mutants. The single marker analysis suggested that the four markers viz., OPC4-K, OPC4-A and OPA15-A are linked with PDI at 0.05 probability level (Table 2).

Table 2: Single marker analysis* for the PDI in sunflower genotypes/mutants

Marker	R^2	Prob > F
OPCO5-B	0.2285	0.0284
OPCO4-K	0.2102	0.0366
OPCO4-A	0.2045	0.0395
OPA15-A	0.2004	0.0418

*The PDI data was used as dependent and the RAPD data generated using the 10 primers and 15 mutants in M_3 and corresponding 3 control genotypes of sunflower and their respective M_2 as independent variables

R^2 values were calculated to explain the variability using these markers in respect of PDI indicate that OPC5-B and OPC5-K could explain 22.8% and 21.0% followed by OPC4-A (20.4%) and OPA15-A (20.0%). Stepwise regression analysis to find the best combinations of markers affecting PDI indicated that OPC5-B, OPC5-

K, OPC5-J, OPA12-D and OPA15-A together explained 80% of PDI (Table 3). Statistical analysis shows that the markers OPC5-B and OPA15-A are associated with PDI (Table 2). Hence these alleles have the potential to be used as markers to explain the variability for PDI (Table 3) traits associated with *Alternaria helianthi* resistance.

Table 3: Stepwise regression analysis* for PDI in sunflower genotypes/mutants

Marker	Partial R^2	Total R^2	Prob > F
OPCO5-B	0.2286	0.2286	0.0284
OPCO5-K	0.2082	0.4368	0.0189
OPCO5-J	0.1583	0.5951	0.0195
OPA12-D	0.1236	0.7187	0.0174
OPA15-A	0.0872	0.8058	0.0203

*The PDI data was used as dependent and the RAPD data generated using the 10 primers and 15 mutants in M_3 and corresponding three control genotypes of sunflower and their respective M_2 as independent variables

One of the most efficient and economical means to manage leaf spot disease is to breed for resistance, but it is limited by the lack of known sources of resistance to *Alternaria helianthi* (Morris *et al.*, 1983). Although significant differences among sunflower lines for reaction to *Alternaria* leaf blight have been reported (Carson, 1985; Das *et al.*, 1998; Sujatha *et al.*, 1997), resistance source that could be used in breeding program has not been identified. At present, all the genotypes and hybrids under cultivation are highly susceptible. Mutation breeding seems to be viable and valuable exercise in obtaining genotypes with resistance or for breaking undesirable linkages in existing genes for disease resistance (Liu, 1990). Molecular marker techniques, especially RAPD (Williams *et al.*, 1990; Uma *et al.*, 2004), have been popular for identifying markers associated with various disease resistance genes. In the present investigation, we made a successful attempt to develop some mutant lines of sunflower resistant to *Alternaria* and markers associated with PDI that will be useful for marker-assisted selection in breeding for resistance to *Alternaria* in sunflower.

ACKNOWLEDGEMENTS

We acknowledge the financial support in the form of Senior Research Fellowship to UKM and research grant to DTP from Board of Research on Nuclear Sciences, BARC, Mumbai (No. 2000/35/5-BRNS/312).

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EVALUACIÓN DE LA HERENCIA DE RESISTENCIA A *Alternaria helianthi* EN GIRASOL UTILIZANDO LOS MARCADORES MOLECULARES

RESUMEN

La alternariosis de la hoja, causada por *Alternaria helianthi* (Hansf.) es una importante enfermedad fúngica de girasol, tanto en La India, como en otros países también. La carencia de las fuentes de resistencia a alternaria conocidos, representa una limitación significante en la selección de girasol. Por ello, hemos sometido la semilla de genotipos de girasol a la radiación gama, con el fin de crear las poblaciones mutantes. Los mutantes fueron elegidos de la generación M_2, sobre la base del porcentaje de presentación de la enfermedad, y luego fueron cultivados en la generación M_3. La DNA genómica de las hojas fue evaluada por el método PCR con la utilización de los primers decamer al azar, y los fragmentos de DNA multiplicados de mutantes, fueron comparados con los controles correspondientes. El análisis de los marcadores individuales y las regresiones escalonadas en relación con el índice de enfermedad, indicó que los alelos OPC5-B, K, J, OPA12-D y OPA15-A erán estrechamente vinculados con la resistencia de *Alternaria helianthi*.

ESTIMATION DE L'APTITUDE DU TOURNESOL À HÉRITER DE LA RÉSISTANCE À l'*Alternaria helianthi* AU MOYEN DES MARQUEURS MOLÉCULAIRES

RÉSUMÉ

La maladie des taches sur les feuilles causée par l'*Alternaria helianthi* (Hansf.) est une mycose importante du tournesol en Inde et ailleurs. L'absence de sources connues de résistance à l'Alternaria est une contrainte majeure dans la culture du tournesol. C'est pourquoi nous avons soumis des semences de génotypes de tournesol à une irradiation gamma pour développer des populations mutantes. Nous avons choisi les mutants dans la génération M_2 selon le pourcentage de l'apparition de la maladie et les avons cultivés dans la génération M_3. L'ADN génomique des échantillons de feuille a été évalué par amplification PCR au moyen des amorces de decamer randomisés et les fragments ADN des mutants ont été comparés à leurs contrôles respectifs. L'analyse des marqueurs individuels et de la régression graduée par rapport à l'index de la maladie a montré que les allèles OPC5-B, K, J, OPA 12-D et OPA 15-A étaient étroitement liés à la résistance à l'*Alternaria helianthi.*