

MUTATION BREEDING IN SUNFLOWER FOR RESISTANCE TO ALTERNARIA LEAF SPOT

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SUMMARY

Genetic variability for resistance to *Alternaria* leaf spot disease (*Alternaria helianthi*) can be induced by radiation or chemical mutagens. The objectives of this study were to create genetic variability in cultivated sunflower and to select lines resistant to *Alternaria* leaf spot. In the first experiment, sunflower seeds of the genotype HA BR 104 were irradiated with 150 and 165 Gy of gamma rays. Seeds were sown in the field at the Embrapa Soybean experimental station, in Londrina, PR, Brazil and M₁ plants were harvested in bulk. M₂, M₃ and M₄ plants were screened for disease resistance under natural infection in the field. Plants were evaluated for *Alternaria* leaf spot symptoms, using a diagrammatic scale from 0 (no symptoms) to 5 (maximum infection). Before flowering, plants showing no symptoms of *Alternaria* leaf spot (grade 0) or less than 5% diseased leaf area (grade 1) were bagged for self-pollination. Self-pollinated plants and open-pollinated plants from 150 Gy and 165 Gy populations with no or mild disease symptoms were selected. In the second experiment, sunflower seeds of the genotypes HA 300 and HA BR 104 were treated with ethyl methanesulfonate (EMS) at 0.015 mol dm⁻³. Selected M₂ and M₃ were screened for disease resistance in the field. From the EMS treatment, 300 M₃ plants with no disease were recovered. All these lines will be tested for combining ability. The best lines will be used for hybrid production.

Key words: *Helianthus annuus*, *Alternaria helianthi*, gamma ray, chemical mutagen, Brazil

INTRODUCTION

Sunflower is an oilseed crop with wide adaptation, due to its tolerance to low temperatures and drought. The seeds have a high oil content with very good quality. This crop represents a new option for Brazilian farmers, considering both agro-economic and market aspects (Castro *et al.*, 1996).

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Diseases are one of the major limiting factors of sunflower production worldwide. The sunflower plant is a known host of over three dozen infectious microorganisms, mostly fungi, which may, under certain climatic conditions, significantly reduce yield and quality (Gulya *et al.*, 1997).

Alternaria leaf and stem spot, seedling blight and head rot, caused by *Alternaria helianthi* (Hansf.) Tubaki and Nishihara, are important diseases occurring on sunflowers in Brazil, especially in the southern part of the country. This pathogen produces dark brown lesions on leaves, petioles, stems, and flower parts that coalesce and form large necrotic areas (Leite, 1997). Chemical control is not recommended because of the difficulty in obtaining complete foliage coverage by aerial application of fungicides.

Therefore, genetic control of the disease through breeding for resistance is highly desirable, because of the economical aspects (Davet *et al.*, 1991). However, the genetic base of cultivated sunflower is narrow and resistance genes are scarce. Resistance to Alternaria diseases has been found in some wild species of *Helianthus*, like *H.hirsutus*, *H.pauciflorus* (= *H.rigidus*) and *H.tuberosus* (Morris *et al.*, 1983; Lipps and Herr, 1986; Davet *et al.*, 1991; Ravikumar *et al.*, 1995). The use of these species as sources of resistance requires interspecific hybridization with *H.annuus*. This is particularly difficult because these wild species are usually tetraploid or hexaploid, while the cultivated sunflower is diploid (Seiler and Rieseberg, 1997).

Wider variability within *H.annuus* would be very useful in order to identify sources of resistance to Alternaria diseases. One way to create genetic variability in cultivated sunflower is to induce mutations by radiation with gamma rays or chemical mutagens. Seed treatment with gamma radiation has been extensively used to increase variability for several characteristics, such as days to flowering, seed weight, seed coat color and oil content in cultivated sunflower (Gupta, 1976; Ivanov and Stamatov, 1976; Khanna and Bapna, 1976; Robles and Lopez, 1977; Sizova, 1976a, 1976b; Stamatov and Ivanov, 1976; Vranceanu and Stoenescu, 1982; Giriraj *et al.*, 1990; Jambhulkar and Joshua, 1999; Encheva *et al.*, 2003). Chemical mutagens, such as ethyl methanesulfonate (EMS), have also been used for this purpose (Vick and Miller, 1996).

The objectives of the present work were to create genetic variability in cultivated sunflower by radiation and chemical mutagens and to select lines resistant to Alternaria leaf spot.

MATERIALS AND METHODS

Mutations induced by radiation

A preliminary trial was performed at the Radiation Genetic Section of Agricultural Nuclear Energy Center, Piracicaba, SP, Brazil (CENA-USP), to define the level

of gamma radiation that should be used for treatment of sunflower seeds. Seeds of the line HA BR 104, from Embrapa Soybean, were irradiated with gamma rays at 100, 200, 300, 400 and 500 Gy, at 30 cm distance during 14.4 min. Each treatment consisted of 10 seeds, with 3 replicates. The irradiated seeds were tested in the greenhouse, with non-irradiated seeds as controls. The seedlings were evaluated for germination, hypocotyl and epicotyl length and plant height.

Subsequently, four thousand sunflower seeds of the line HA BR 104 were irradiated with gamma rays 180 Gy for mutagenic treatment. The irradiated and non-irradiated seeds were sown in the field, at the Embrapa Soybean experimental station, in Londrina, PR, Brazil, in order to examine the M_1 plants.

Another trial was carried out in June 1996, when two lots of eight thousand seeds of the same line were each irradiated with 150 or 165 Gy of gamma rays. The M_1 seeds were sown in the field to generate at least four thousand plants, which were self-pollinated. About 300 non-irradiated seeds were sown as controls. Seedlings and plants were evaluated for survival, chlorosis, plant height at harvest, sterility and number of seeds per head. All M_1 plants were harvested in bulk.

On January 1997, the next generation (M_2) was sown in a dense stand of about 70,000 plants ha^{-1} in order to provide conditions for high incidence of *Alternaria* leaf spot. No inoculation with *A. helianthi* was performed and disease occurred by natural infection of the plants in the field. The plants were irrigated twice a week, to provide the humidity required by the pathogen to spread. The plants were evaluated for *Alternaria* leaf spot symptoms using a diagrammatic scale from 0 (no symptoms) to 5 (maximum infection). Before flowering, the plants showing no symptoms of *Alternaria* leaf spot (grade 0) or less than 5% diseased leaf area (grade 1) were protected for self-pollination. These plants were evaluated periodically for occurrence of disease symptoms. The plants with low disease severity were harvested individually, to produce M_3 lines. At harvest time, open-pollinated plants that showed few disease symptoms were collected, in order to form M_3 half-sib families.

In January 1998, M_3 lines from self-pollinated plants or open-pollinated plants from both radiation levels' populations were sown for evaluation of disease resistance and to get the M_4 seeds, at the Embrapa Soybean experimental station, in Londrina, PR.

The M_4 lines from self-pollinated plants of 150 and 165 Gy radiation levels were sown on two dates in Londrina, PR (December 8, 1998 and February 2, 1999) and on February 2, 1999 in Planaltina, DF, for evaluation of disease resistance, agronomic characteristics and oil content, compared with the control plants. These two locations represent different regions for sunflower production in Brazil.

Mutations induced by chemical mutagens

The mutagen selected for induced mutations was ethyl methanesulfonate (EMS). A preliminary trial was carried out to determine the appropriate concentration range of EMS for mutagenesis in sunflower. Sunflower seeds of the genotypes HA 300, from USDA, and HA BR 104, from Embrapa Soybean, were treated with EMS at 0 (control), 0.010 mol dm^{-3} , 0.015 mol dm^{-3} and 0.020 mol dm^{-3} . Each lot consisted of 13 seeds, with four replicates. Seeds were pre-treated with potassium

phosphate buffer pH 8.0 for 8 h. They were then treated with EMS solution for 16 h, rinsed and sown in greenhouse. The plants of the genotype HA 300 were evaluated for vigor and plant height two weeks after emergence.

Another trial was performed using the selected EMS concentration for mutagenic treatment of sunflower seeds. Ten thousand seeds of the line HA BR 104 and fourteen thousand seeds of the line HA 300 were treated with EMS at $0.015 \text{ mol dm}^{-3}$ for 16 h. Untreated seeds were used as controls. After treatment, seeds were sown in the field on July 9, 1997, at the Embrapa Soybean experimental station, in Londrina, PR, in order to produce M_1 plants. The seedlings and plants were evaluated for survival, chlorosis, plant height at flowering time and harvest, sterility and number of seeds per head. All M_1 plants were harvested in bulk in November, 1997.

In January 1998, the next generation (M_2) was sown at a dense stand of about 60,000 plants ha^{-1} , in order to provide conditions for high incidence of *Alternaria* leaf spot. The plants were irrigated twice a week, to increase the humidity required by the pathogen to spread. The plants were evaluated for *Alternaria* leaf spot symptoms, using the diagrammatic scale previously described. Before flowering, the plants showing no symptoms of *Alternaria* leaf spot (grade 0) or less than 5% diseased leaf area (grade 1) were bagged for self-pollination and were evaluated periodically, to observe the disease evolution. The plants with low disease severity were harvested individually on May 8, 1998. From these plants, M_3 lines were obtained.

The M_3 lines from self-pollinated plants of EMS treatment were sown on two dates (December 8, 1998 and February 2, 1999) in Londrina, PR, and on February 2, 1999 in Planaltina, DF, for evaluation of disease resistance, agronomic characteristics and oil content, compared with the control plants. *Alternaria* disease occurred by natural infection of the plants.

RESULTS AND DISCUSSION

Mutations induced by radiation

The preliminary trial performed in the greenhouse for determination of the desired level of gamma radiation for sunflower seeds is summarized in Table 1.

Table 1: Means of germination rate, hypocotyl and epicotyl length and height of seedlings, following treatments with gamma radiation

Dose (Gy)	Germination rate ^a (%)	Hypocotyl length ^a (mm)	Epicotyl length ^a (mm)	Plant height ^a (mm)
0	86.7	22.7	36.0	59.7
100	90.0	19.8	32.9	52.7
200	100.0	10.9	16.5	37.4
300	77.0 ^b	5.7	-	-
400		-	-	-
500		-	-	-

^a means of three replications with 10 plants

^b plants without leaves

The seeds survived up to the 200 Gy level treatment. However, there was a marked reduction in plant height (about 37%). Based on these results, the dose of 180 Gy was chosen for further experimentation.

M₁ seedlings from the 180 Gy treatment, evaluated in the field experiment, showed low germination rate (lower than 50%) and there was high level of plant sterility, indicating that this dose was still too high.

Table 2: Means of survival, sterility, plant height, and seeds per head of sunflower M₁ seedlings and plants, following treatments with gamma radiation

Dose (Gy)	Survival ^a (%)			Sterility ^b (%)	Plant height (cm)	Number of seeds per head
	15 DAE	30 DAE	At harvest			
0	73.8	74.5	67.1	7.3	67.80	88
150	48.4	43.9	28.5	9.6	48.72	35
165	50.4	50.2	34.3	41.4	47.89	29

^a means of 283, 3395 and 3401 plants for 0, 150 and 165 Gy, respectively

^b means of 190, 967 and 1166 plants for 0, 150 and 165 Gy, respectively

DAE = days after emergence

A reduction in the M₁ seedling emergence rate and survival was observed for both 150 and 165 Gy radiation treatments (Table 2). No plants showed chlorosis, but sterility was markedly increased by the 165 Gy dose. Reductions also occurred in plant height and number of seeds per head for both radiation levels. Most control plants were 60-90 cm high, while M₁ plants from the 150 and 165 Gy treatments were considerably shorter (35-60 cm). The number of seeds per head was reduced in most of the treated plants, ranging from 1 to 51 seeds per head. These results indicated that the radiation levels used for sunflower seeds were still too high.

In the M₂ generation, the plants were evaluated for Alternaria leaf spot and those showing no symptoms or less than 5% diseased leaf area were protected. Unprotected plants that showed less symptoms were also collected. The selected plants were listed in Table 3. Some plants showing head deformation were collected to evaluate the maintenance of this character in the subsequent generation.

Table 3: Self-pollinated and open-pollinated M₂ plants evaluated for disease resistance and head deformations following treatments with gamma radiation

Dose (Gy)	Number of evaluated plants	Disease resistance plants harvested		Open-pollinated with head deformations
		Self-pollinated	Open-pollinated	
150	8406	2	25	10
165	5552	3	28	10

After screening M₂ plants for disease resistance, the next generation, M₃ lines, from self-pollinated plants and open-pollinated plants from both radiation levels were sown and evaluated for Alternaria leaf and stem spot under high disease natural conditions in Londrina, PR. The disease severity was evaluated using the same scale used in the M₂ generation. Agronomic characteristics were not evaluated because lines were still heterogeneous. Plants of the M₃ lines were selected for Alternaria disease resistance (Table 4).

The M₄ lines were evaluated in two sowing dates in Londrina, PR and in one trial in Planaltina, DF. In both locations, the climatic conditions were very favorable for disease epidemics, and all the M₄ lines showed high severity of Alternaria leaf spot. Based on the susceptibility of these lines, they were considered not useful for sunflower breeding programs for disease resistance.

Table 4: M₃ lines tested and plants selected for Alternaria disease resistance, following treatments with gamma radiation

Dose (Gy)	Self-pollinated lines		Open-pollinated lines		Open-pollinated with head deformations	
	Evaluated lines	Selected plants	Evaluated lines	Selected plants	Evaluated lines	Selected plants
150	2	1	25	10	10	6
165	3	1	28	29	10	4
Total		2		39		10

Mutations induced by chemical mutagens

Despite the reduction in plant height (12.5%), plants of the EMS 0.015 mol dm⁻³ treatment showed good development (Table 5). Seeds of HA BR 104 emerged poorly, but their physiological aspects were normal. Based on these results, the chosen concentration for EMS treatment was 0.015 mol dm⁻³.

Table 5: Means of plant height of HA 300 sunflower seedlings, following treatments with EMS

EMS concentration	Plant height (cm) ^a	Reduction (%)
0 (control)	11.2	-
0.01 mol dm ⁻³	10.8	3.6
0.015 mol dm ⁻³	9.8	12.5
0.02 mol dm ⁻³	8.3	25.9

^a means of 4 replications with 13 plants each

In the second trial, the EMS treatment reduced the M₁ seedlings survival for both HA BR 104 and HA 300 (Table 6). Plants showing chlorosis died three days after emergence.

Table 6: Means of survival, sterility, plant height, and seeds per head of HA BR 104 and HA 300 sunflower M₁ seedlings and plants, following treatments with EMS

Genotype	EMS	Survival ^a (%)			Sterility ^b (%)	Plant height (cm) ^c		Seeds per head ^d
		5 DAE	21 DAE	31 DAE		flowering	harvest	
HA BR 104	0	52.00	52.00	52.00	2.00	73.39	72.35	-
HA BR 104	0.015 mol dm ⁻³	41.17	41.63	41.40	1.74	79.38	81.80	50.00
HA 300	0	82.00	83.00	82.00	0.00	123.70	123.03	-
HA 300	0.015 mol dm ⁻³	63.95	65.66	63.37	0.00	108.50	107.87	58.56

^a means of 200, 7800, 200 and 12800 seeds, respectively for HA BR 104 (0 and 0.015 mol dm⁻³) and HA 300 (0 and 0.015 mol dm⁻³)

^b means of 52, 3229, 82 and 8111 plants, respectively for HA BR 104 (0 and 0.015 mol dm⁻³) and HA 300 (0 and 0.015 mol dm⁻³)

^c means of 300 plants

^d means of 200 plants

DAE = days after emergence

The mutagenic agent did not cause sterility; the sterility observed in plants of the genotype HA BR 104 was natural, because the untreated plants showed the same sterility percentage as the EMS-treated population (Table 6). Plant height of HA 300 treated with EMS was reduced. For the genotype HA BR 104, this difference was not significant; most of HA BR 104 control plants were 65-95 cm high, while M_1 plants were 70-95 cm. For HA 300, the control plants' height was 120-140 cm and M_1 plants' height was lower (95-135 cm). The number of seeds per plant was assessed only for EMS-treated population, because the untreated control seeds were pooled after harvesting.

In the M_2 generation, the plants were evaluated for Alternaria leaf spot as previously described and plants showing no symptoms or less than 5% diseased leaf area were protected and harvested individually. The remaining open-pollinated plants that showed low disease severity after flowering were discarded because of the low genetic gain showed by this procedure in the previous experiment of inducing mutation by gamma radiation. The selected plants were listed in Table 7.

Table 7: M_2 plants selected for Alternaria disease resistance following treatments with EMS

Genotype	EMS	Evaluated plants	Self-pollinated plants selected
HA BR 104	0.015 mol dm ⁻³	22080	16
HA 300	0.015 mol dm ⁻³	6720	393

The M_3 lines from self-pollinated plants from both HA BR 104 and HA 300 were sown in Londrina and Planaltina and evaluated for Alternaria leaf and stem spot under natural conditions for high disease occurrence. The disease severity was evaluated using the same scale used for the M_2 plants. Plants of M_3 lines with low disease severity were selected.

From the EMS treatment, 300 M_3 plants with no disease symptoms were recovered. The agronomic characteristics and oil content were not evaluated because the number of seeds per head was insufficient.

The newly developed lines were characterized by increased Alternaria leaf spot resistance when evaluated under natural infection in the field. The cytoplasmic male sterility has been introduced in the best lines. All these lines will be tested for combining ability and can be used in the sunflower breeding programs for hybrid production.

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MUTACIÓN PARA MEJORAR GIRASOL CON RESISTENCIA A LA ALTERNARIOSIS DE LA HOJA

RESUMEN

La variabilidad genética para obtener resistencia a la Alternariosis de la hoja (*Alternaria helianthi*) puede ser inducida por radiación o con agentes mutagénicos químicos. Los objetivos de este trabajo fueron crear variabilidad genética en girasol cultivado y seleccionar líneas resistentes para Alternariosis de la hoja. En el primer experimento, semillas de girasol del genotipo HA BR 104 fueron irradiadas con 150 y 165 Gy de rayos gamma. Las semillas fueron sembradas en el campo experimental de la Embrapa Soja, en Londrina, PR, Brasil y las plantas M₁ se cosecharon en "bulk". Plantas M₂, M₃ y M₄ se seleccionaron por resistencia a la enfermedad, en condiciones naturales, a campo. Se hicieron evaluaciones de los síntomas provocados por *Alternaria*, utilizando una escala de 0 (sin síntomas) a 5 (máximo de la enfermedad). Antes de florecer las plantas que no presentaron síntomas de Alternariosis (grado 0) o menos que 5% del área de la hoja afectada (grado 1) fueron embolsadas para auto polinización. Se seleccionaron plantas auto-polinizadas y de polinización abierta de las poblaciones provenientes de los tratamientos con 150 Gy y 165 Gy sin síntomas o con síntomas leves. En el segundo experimento, semillas de girasol de los genotipos HA 300 y HA BR 104 fueron tratados con etil metanosulfonato (EMS) 0.015 mol dm⁻³. Los materiales M₂ y M₃ fueron seleccionados para resistencia a esta enfermedad en el campo. En los tratamientos con EMS, se recuperaron 300 plantas M₃ sin la enfermedad. Todas estas líneas serán ensayadas por habilidad de combinación. Las mejores líneas serán utilizadas para la producción de híbridos.

MUTATION POUR L'AMELIORATION DE LA RESISTANCE DU TOURNESOL VIS-A-VIS DE LA ROUILLE DES FEUILLES

RÉSUMÉ

La variabilité génétique pour la résistance vis-à-vis de la rouille des feuilles (*Alternaria helianthi*) peut être obtenue par traitements mutagènes physiques ou chimiques. Les objectifs de cet étude ont été de créer variabilité génétique dans des variétés cultivées de tournesol et de sélectionner les variants résistants à *A. helianthi*. Dans le premier essai, des semences du génotype HA BR 104 de tournesol ont été irradiées avec 150 et 165 Gy du rayon gamma. Les semences ont été semées dans les champs de la station expérimentale Embrapa-Soja, située à Londrina, PR, Brazil et les plantes M₁ ont été récoltées par bulk. Plantes M₂, M₃ et M₄ ont été sélectionnées vis-à-vis de la résistance à la maladie dans des conditions de champs sous infection naturelle. La sévérité des symptômes a été évaluée par une échelle de notation de 1 (sans symptômes) à 5 (maladie maximale). Avant la floraison, les plantes sans symptômes (degré 1) et celles avec moins de 5% de sévérité (degré 1) ont été ensachées pour la auto-polinisation. Les plantes sans ou avec un peu de symptômes provenant des populations 150 Gy et 165 Gy des polinisation ouvertes ou fermées ont été sélectionnées. Dans le deuxième essai, des semences des génotypes HA 300 et HA BR 104 de tournesol ont été traitées avec ethyl methanesulfonate (EMS) a 0.015 mol dm⁻³. Les populations M₂ et M₃ ont été sélectionnées vis-à-vis de la résistance au champs. Troiscent plantes M₃ sans symptômes ont été réperées. Toutes les lignes seront testées pour l'habilité de combinaison. Les meilleures seront utilisées pour la production des hybrides.

