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MUTAGENIC TREATMENTS PERFORMED ON SEEDS OF A SUNFLOWER HYBRID VARIETY WITH THE PURPOSE OF OBTAINING BIFENOX OR GLYPHOSATE RESISTANT MUTANTS

André Bervillé[1], Jacky Delbut & Robert Bedergat

INRA DIJON – Station d'Amélioration des Plantes, BV 1540, 21034 DIJON Cédex, France

SUMMARY

Mutagenic treatments were performed on F1 sunflower seeds with gamma rays (100 Gy, 200 Gy, 300Gy or 400 Gy dose) and with 0.2% ethyl methyl sulfonate. The screening for bifenox or glyphosate resistant progenies led to 18 M2 homogeneous bifenox unaffected (resistant) progenies and 113 progenies segregating glyphosate partially resistant plants, respectively. The ratios of progeny numbers containing – bifenox homogeneous unaffected /segregant resistant plants, and – segregant partially resistant/homogeneously sensitive plants, suggested a mutational event towards a recessive allele. In the field, spraying with glyphosate appeared inefficient to study the mode of inheritance of the resistance. Therefore we developed an *in vitro* assay allowing us to investigate four progenies only segregating partially glyphosate resistant plants. About ten per cent of the individuals of these four progenies survived the *in vitro* assay while no control plant did. Glyphosate resistance was controlled for four generations of the progenies. The level of resistance against glyphosate was reproducible but low *in vitro* (10 μ Roundup/l) or in field (360 g/ha) so that further work will be done to check a possible duplication of the EPSP synthase gene.

Key words: bifenox resistance, glyphosate resistance, hebricide resistance, *Helianthus annuus*, mutagenesis sunflower

INTRODUCTION

Sunflower varieties are now widespread in Europe especially in France, Spain and Italy. One of the most important cultural problems is to prevent weed development. The use of herbicides has been recommended. But sunflower varieties are sensitive to most herbicides. In France, CETIOM[2]: (1990) recommends herbicides at presowing and postsowing time, and on seedlings. The cost of these three treatments is high so that the introduction into sunflower of a herbicide resistance gene appears to be a useful objective, (Freyssinet, 1986).

Two methods have been used to introduce herbicide resistant genes into plants. Direct transformation of plant genome has been performed, for example, on sugar beet, tomato, and Brassica (Bedbrook et al., 1988), but this method is not yet efficient on sunflower. The induction of a herbicide resistant gene is a second method and it has already been performed on *Arabidopsis*.

1 to whom correspondence should be sent

2 Centre d'Etude Technique et Industriels des Oléagineux Métropolitains

With herbicide resistance, most mutants have been obtained for several species through tissue culture without a preceding mutagenic treatment. In regenerated plantlets, herbicide resistant individuals were obtained (Amrhein et al., 1983; Nafziger et al., 1984; Smith et al., 1986; Shah et al., 1986). In *Arabidopsis thaliana*, a mutagenic treatment of seeds with ethyl methyl sulfonate resulted in resistance to sulfonylurea (Haugh and Somerville, 1986.

MATERIALS AND METHODS

Mirasol, a hybrid from Cargill–France, obtained by crossing two lines HA89A and RHA274, was used throughout the experiments while the two lines and HA89B were used as controls (A means CMS cytoplasm, B means maintainer of male sterility).

Field assays

Dry seeds (2000) were exposed to gamma rays (^{60}Co source, 240 Gy/h) so that they received either a 100 Gy, 200 Gy, 300 Gy. or 400 Gy dose. Dry seeds (5000) were also soaked in 0.2% ethyl methyl sulfonate (EMS) for 16 h.

The seeds (M1 generation) were planted in the field in order to obtain M1 plants. About 1650 M1 irradiated seeds per dose and 5350 seeds treated with EMS were planted in the field. A family corresponds to the progenies of one M1 plant. Plants were self-pollinated under preservative bags and all further crosses were controlled.

Herbicide treatments

Bifenox [5-(2,4-dichlorophenoxy)-2nitrobenzoate] inhibits photosynthetic electron transport (Bugg et al., 1980). It has been reported to cause scorching on young sunflower plants at the B3-B4 leaf stage when sprayed at 1.5 kg/ha (Y. Regnault, personal communication). Therefore, we looked for plants without scorching symptoms at 3 kg/ha.

Glyphosate [(phosphonomethy-amino)-2-acetic acid)] inhibits the aromatic amino-acid pathway at the 5-enolpyruvylshikimate-3-phosphate synthase step (EPSP synthase). Resistance to glyphosate has occurred either by EPSP synthase gene amplification (Steinrücken et al., 1986;Smith et al., 1986), or by mutation within the EPSP synthase gene (Amrhein et al., 1983). We looked in sunflower for resistance to a dose of 0.36 kg/ha which killed the control plants. That dose killed all the weeds in the field under assay, whatever their developmental stage.

Herbicide treatments were performed on M2 progenies (35 seeds per M1 plant) with 3 kg/ha of bifenox kindly provided by Rhône Poulenc Agochimie and 200 l/ha of a 0.5% Roundup solution (commercial product from Monsanto containing 360 g/l of glyphosate). The herbicide effects were observed 2, 9 and 18 days later. In the field with bifenox treatments we numbered the homogenous sensitive progenies, those without any symptoms and those containing both sensitive and resistant plants. In the field with Roundup treatment, only progenies with 2 or more surviving individuals were multiplied by selfing or by crossing with HA89B pollen.

To determine the level of glyphosate resistance *in vitro*, seeds were allowed to germinate on a mineral medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose, 100 mg/l myoinositol, 200 mg/l L-glutamine, 0.8% Agar-Agar and glyphosate (10 μl/l of Roundup). After 40 days on this medium, the surviving plantlets were

transferred to the same culture medium, except glyphosate, with 1 mM L-tyrosine and 1 mM L-phenylalanine. Twenty days later plantlets were transferred to the glasshouse.

RESULTS

Field assays

M1 plants were selfed in the summer of 1985 and seeds were separated in aliquote fractions of 35 seeds which were sown the next year. About 20% of the M1 plants did not produce any seeds because of abnormalities in their development: male sterility, female sterility, abortion of the head, so that 3991 progenies only were obtained. But 3028 progenies contained more than 105 seeds, allowing three replicates of 35 seeds (Table 1). The M2 progenies with insufficient seeds were sown in 1987 to obtain M3 plants which were selfed or sib-crossed. In 1986, two replicates of the 3028 progenies were sown in the field. One field was sprayed with bifenox and since we expected only slight damage, it was kept as a control for plant observation. The other field was sprayed with gliyphosate.

Table 1. Numbers of progenies, with more than one glyphosate resistant plant, induced with four gamma ray and 0.2% EMS.

Mutagen	M2 progenies obtained with more than 105 seeds	Progeniesn with more than one glyphosate resistant plant	
gamma rays			
100 Gy	377	31	8.22%
200 Gy	946	46	4.8%
300 Gy	585	17	2.89%
400 Gy	211	7	3.3%
EMS 0.2%	909	19	2.09%

a) In vivo assays

The number of progenies without any damage caused by bifenox was 18 out of 3028 M2 progenies (0.6%). Two of these 18 progenies were verified by selfing to be fixed for bifenox tolerance in the next generation. About 400 progenies displayed segregation for affected or unaffected plants while 2610 progenies were homogeneously affected.

The number of progenies with more than one surviving glyphosate treated plant is indicated in Table 2. The individuals which survived the glyphosate spray, and reached the flower, were selfed if male-fertile, or sib-crossed if male-sterile. Table 2 indicates the individuals surviving the glyphosate spray.

Table 2. Progenies screened in the field with several glyphosate resistant individuals.

Year observation in field	observed	Progenies with several resistant individuals
1986	M2	23
1987	M2	2
1988	M2	11
1988	M3	4
1989	M2	12

The following assays in the field revealed difficulties due to the reproducible climatic conditions for the evaluation of the glyphosate resistance. Consequently, the assays were carried out *in vitro* once the standard conditions had been determined.

b) In vitro assays

The development of seeds from Mirasol, HA89B and RHA274 was followed on 169 medium. The optimal glyphosate concentration was determined by comparison of seedling development in 169 plus either 1, 2, 5, 10, 15, 20, 30, or 50 μ of glyphosate per l. We retained 10 μ of the Roundup commercial stock per liter for all further uses. Seeds from field resistant plants and from control plants were compared on the same basic medium without and with glyphosate. All control plants were killed by glyphosate within 15 days. Among the 113 field resistant progenies (24 seeds) in the medium containing 10 μl/l of Roundup, some individuals survived up to 120 days, while the others were killed within 15 days. Three assays of 24 seeds per progeny were done. The sums of the three assays are displayed in Table 3. Among 113 progenies with glyphosate resistant plants, retained from the screening in the field, only 4, in the *in vitro* assays, repeatedly displayed glyphosate resistant seedlings. We considered further that the *in vitro* assay revealed the progenies with true resistant plants in comparison with the field assay where plants may escape the glyphosate spray.

Table 3. Progenies screened *in vitro* for glyphosate resistant plant.

progenies screened *in vitro*	progenies with resistant plants	M_2 progeny code	ratio resistant sensitive per M_3 progenies
113	4		
		109	10/60
		2804	7/60
		2448	4/32
		4146	5/36

The transfer of the resistant plants to normal conditions was difficult, possibly because of gliphosate accumulation in their tissue. Thus, we first transferred the plants to the same basic medium supplemented with 1 mM L. tyrosine and 1 mM L. phenylalanine. The growth of plantlets was clearly improved on this medium, so that after 20 days they were transferred to the glasshouse.

After 100 days of culture it took the plants one month to flower. Their average height was 20 cm +_ 10 cm. Among the 4 progenies which led to resistant individuals after 40 days, we obtained 26 plants (table 3). Five of them produced seeds which will be studied in the spring of 1992. The segregation ratios obtained may be biased in these experiments sience we lost plants.

DISCUSSION

The mutagenic treatment performed, on a large scale, on the sunflower hybrid Mirasol (Cargill, France) HA89A x RHA274 led to the identication of mutants which affect the herbicide resistance.

The screening with bifenox led to several progenies ready to be used directly in breeding programs. Our data are in agreement with the mutation of one recessive allele

in M1 seeds leading to homogeneously unaffected M2 progenies. We will look for a possible additive effect of alleles induced in the different mutant families.

The screening with glyphosate in the field was efficient and led to 2% (62/3028) progenies in the field with resistant individuals. After repetitive *in vitro* screening of the same M2 or M3 progenies, only 4 numbers (0.13%) were kept as resistant. The method in the field was not effective to allow the study of the inheritance of resistance, which has already been pointed out (Pelletier, 1986). *In vitro*, we verified that among the same progenies, we obtained repeatedly resistant individuals. We cannot check any genetic model of resistance since we lost plants between tubes and the glasshouse.

Nevertheless the level of resistance is low in comparison with resistant manipulated plants of Petunia (Steinrücken et al. 1986) or tomato (Fillati et al. 1987). In order to improve the level of resistance crosses were done between plants expected to carry a favorable allele. This low level of resistance suggests that the genetic event leading to the glyphosate resistant trait could be a duplication of the EPSP gene. This event will be looked for with a convenien probe of the EPSP gene.

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RESUMEN

Tratamientos mutàgenos por exposición a rayos gamma (a dosis de 100 Gy, 200 Gy, 300 Gy o 400 Gy) y a 0.2% de etil metil sulfonato fueron efectuados sobre semillas F1 de girasol. La selección de progenies resistentes a bifenox o a glyfosato condujo respectivamento, a la obtención de 18 progenies M2 homogéneas no afectadas por el bifenox (resistentes) y 113 progenies segregantes, compuestas de plantas parcialmente resistentes a glyfosato. La relación entre progenies homogéneas no afectados/progenies parcialmento resistente a bifenox y entre progenies parcialmento resistentes/progenies homogéneas sensibles a bifenox sugiere un evento mutacional hacia un alelo recesivo. Los tratamentos de asperción con glyfosato realizados a compo no fueron eficaces para el estudio de la herencia de la resistencia. Por eso hemos desarrollado un ensayo *in vitro* que nos permitió estudios cuatro progenies que segregan sólo plantas parcialmente resistentes a glyfosato. Aproximadamente diez po ciento de los individuos de estas cuatro progenies sobrevivió al ensayo *in vitro* mientras que ninguna planta del control lo hizo. La resistencia a glyfosfato fue reproductible pero baja en los ensayos *in vitro* (10 μ Roundup/l) o a compo 360 g/ha); por lo tanto, se realizaràn nuevas experiencias para probar la hipótesis de una posible duplicatión del gene de la EPSP sintetasa.

RESUMÉ

Des traitements mutagènes ont été réalisés sur des graines d'un hybride F1 de tournesol avec des rayons gamma (dose de 100Gy, 200Gy, 300Gy ou 400Gy) ou avec 0.2% de methyl sulfonate d'éthyle. Le tri pour les descendances contenant des plantes résistantes soit au glyphosate soit au bifenox a conduit à 18 descendances entièrement non affectées (résistantes) par le bifenox et 113 descendances qui ségrègent des plantes partiellement résistantes et sensibles. Après le traitment bifénox, les rapports du nombre de descendances entièrement non affectées/descendances en ségrégation, et des descendances contenant en ségrégation des plantes résistantes et sensibles/descendances entièrement sensibles, suggèrent la mutation d'un gène vers un allèle de résistance récessif. Dans le champ, le traitment au glyphosate s'est révélé insatisfaisant pour étudier le mode d'hérédité de la résistance. Nous avons donc mis au point un test *in vitro* permettant l'étude des descendances contenant en ségrégation les individus partiellement résistants et entièrement sensibles. Environ 10% des individus survivent au test *in vitro* alors qu'aucune plante témoin ne survit. La résistance au glyphosate est apparue dans quatre descendances successives. Le niveau de résistance est toutefois faible mais reproductible *in vitro* (10 μl Rondup/l) ou au champ (360 g/ha) de telle sorte que notre travail futur sera de vérifier si le gène de l'EPSP synthase n'a pas été dupliqué.