

## Use of imidazolinone tolerance to produce male-sterile testers in sunflower breeding programs.

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### ABSTRACT

- Line x tester crosses are used to test the combining ability of sunflower lines. To test the combining ability of restorer lines, experimental hybrids can be easily developed using a cytoplasmic male sterile (CMS) system. Testing the combining ability of the maintainer lines is much more difficult, even though some procedures were developed to male-sterilize the restorer lines (e.g.: gibberellic acid applications, nuclear male sterility linked to hypocotyl color and CMS sources different than PET1). Here we describe an efficient and effective method for producing sterile tester lines based on organ specific changes in gene expression of the sunflower *Ahasl* genes following herbicide application during early reproductive growth stages.
- Fifteen maintainer inbred lines (five genotypes at the *Ahasl1* locus —susceptible, homozygous and heterozygous CLPlus and Imisun— in three different genetic backgrounds) were used. In addition, two heterozygous lines for the *Ahasl1-3* allele were produced by crossing homozygous tolerant and susceptible near isogenic lines (NILs). These lines were produced as a result of the conversion process of susceptible wild type parental lines to their Clearfield Plus (*Ahasl1-3/Ahasl1-3*) counterparts. All these materials were treated with imazapyr at 40 to 80 g a.i. ha<sup>-1</sup> doses during V8 to R1. During flowering, morphology of the heads of the treated plants was assessed as well as their pollen production and viability. Viability of the ovules was assessed by crossing each induced male sterile plant of the heterozygous restorer lines with a fertile line. Seeds from these crosses were planted and sprayed in V2-V4 with imazapyr under greenhouse conditions to determine the percentage of tolerant plants.
- Susceptible genotypes were killed by all of the treatments. Heterozygous and homozygous Imisun lines showed severe damage of the head and/or complete male and female sterility. Homozygous CLPlus lines showed no phytotoxicity symptoms and were male and female fertile. Heterozygous CLPlus lines, on the other hand, were consistently male sterile after herbicide applications and did not show any other injury. When pollinated by pollen from untreated susceptible lines, they produced a variable amount of seed which, on average, was half the number obtained from the self-pollinated untreated control plants. Plants of the progeny obtained from the induced male sterile CLPlus heterozygous plants crossed with a susceptible line were 98 to 100% tolerant to imazapyr.
- These results are consistent with previous observations regarding differences in tolerance to imidazolinone applications between Clearfield Plus and Imisun genotypes. However, these results also highlight the organ specific patterns of gene expression of the sunflower *Ahasl* genes. In fact, herbicide applications during early reproductive stages of development are well tolerated by heterozygous CLPlus plants but they result in a failure of the anthers to fully develop, rendering the plant male sterile. Due to gametophytic segregation on the female side, instead of a normal 1:1 ratio of tolerant to susceptible ovules, almost all of the female gametophytes carry the tolerant allele. These results allowed us to develop a system of male-sterilization of restorer testers. This new system involves obtaining heterozygous *Ahasl1-3* lines, male sterilizing them using imidazolinone herbicide spray at V8-R1 and multiplying them by crossing the male-sterilized heads with the susceptible restorer lines.
- The method described in this work allows breeders to test the combining ability of maintainer lines in early stages of development. Previously this was done by backcrossing to a sterile CMS source, which is laborious and time consuming. This new method can be used with any type of germplasm. It confers greater flexibility in application timing (compared to gibberellic acid treatments), allows the testing of fertile hybrids (a main difference to the system that relies on CMS testers) and permits a rapid change of the genetic background of the tester (a difference to systems based on nuclear male sterility). Moreover, it can be used to develop any type of recurrent selection method in the breeding program, and increase the frequency of herbicide tolerant plants by gametophytic selection.

**Keywords:** Breeding, Clearfield Plus, CLPlus, gametophytic selection, imidazolinone, tester.

## INTRODUCTION

The final value of a sunflower inbred line can only be determined by testing its general and specific combining ability in hybrid combinations. The most common testers are inbred lines that are being used in commercial hybrids. Miller et al. (1980) and Domínguez and Fernández-Martínez (1987) found that inbred lines are effective in identifying lines with the best combining ability. Evaluation for combining ability often begins at the F4 or F5 generation, but a system of early generation testing beginning after the first generation of selfing was reported to be effective in identifying lines with good combining ability (Shein, 1978).

In early studies, most hybrid seed was produced by hand pollinations without emasculation or by natural crossing in isolated plots. Crossing percentages varied widely and greatly influenced the performance of the test-cross hybrids (Unrau, 1947; Putt, 1962). Subsequent methods involved the use of cytoplasmic or genetic male sterility or the induction of male sterility by gibberellic acid. These methods allow for complete hybridization of lines and hence provided a greater precision in estimating combining ability. Vranceanu (1970) used a monogenic male-sterile line as a female parent to test for combining ability. Chemical emasculation by use of gibberellic acid was originally shown by Schuster (1956) to be effective in causing male sterility, and it was extensively used in producing test crosses between inbred lines and various tester parents (Anashchenko, 1974; Skoric, 1988). However, inbred lines may show different responses to gibberellic acid requiring higher or lower concentrations and earlier or later application times (Piquemal, 1970). Various negative effects such as incomplete male sterility, reduced female sterility, and stem elongation have been associated with the use of gibberellic acid, depending on concentration and timing of application (Miller 1987). An alternative procedure is the rapid conversion of maintainer lines to cytoplasmic male sterility and subsequent production of hybrid seed by hand pollination or isolated crossing blocks. Genetic male sterility linked to a marker gene was also used for the same purpose. In this sense, Miller (1992) released two R-lines (NMS274 and NMS801) in which the genetic male-fertility is linked to a gene for anthocyanin pigmentation, thus allowing for easy identification of male-sterile plants prior to flowering. Currently, evaluation of the combining ability of a potential new R-line usually involves the use of a cytoplasmic male-sterile line (A-line). Cytoplasmic male-sterile lines, including cms sources different from PET1, also are being used as testers of new B-lines. However, in this last case, the obtained experimental hybrids will be sterile, so it is important to include pollen-producing hybrids in the performance trial to ensure adequate pollination (Fick and Miller, 1997; Fernández-Martínez et al., 2009).

Different traits for herbicide tolerance in sunflower were developed during the last decade, which are based on the expression of different alleles at the *Ahas11* locus (Sala et al., 2012). In fact, this locus is the only member of the family of AHAS genes in sunflower where all the induced and natural mutations for herbicide tolerance were described thus far, and also it is the most polymorphic (Kolkman et al., 2004; Hawley, 2005) and the most preponderant in the sunflower EST database (Kozik et al., 2002). This suggests that the *Ahas11* gene is the gene family member that encodes the AHAS enzyme with essential housekeeping functions in sunflower and that it is predominantly expressed in tissues affected by herbicide treatment. Furthermore, previous observations in experiments designed to identify ontogenetic changes in herbicide tolerance indicated that there exist tissue-specific changes in gene expression leading to male-sterility in certain tolerant genotypes. For this reason, we hypothesized that a male-sterilization system could be developed based on the adequate manipulation of herbicide treatments, plant zygosity and development stages.

Here we describe an efficient and effective method for producing sterile tester lines based on organ specific changes in gene expression of the sunflower *Ahas1* genes following herbicide application during early reproductive growth stages.

## MATERIAL AND METHODS

Three maintainer susceptible lines (B7, B8, and B9) together with their homozygous CLPlus and Imisun versions were used. In addition, the heterozygous lines for each trait and genetic background were also obtained. Ten plants of the resulting 15 lines (five genotypes at the *Ahas11* locus —susceptible, homozygous and heterozygous CLPlus and Imisun— in three different genetic backgrounds) were sown under field conditions and treated with imazapyr at 40 and 80 g a.i. ha<sup>-1</sup> doses during V8 and R1 stages of development (Schneider and Miller, 1981). During flowering, these plants were assessed for the morphology of the head as well as for their pollen and seed production in comparison with five untreated plants of each line. In addition, two different inbred lines heterozygous for the *Ahas11-3* allele were produced by crossing homozygous tolerant *Ahas11-3/Ahas11-3* inbred lines (R720CLPlus and

R754CLPlus) with susceptible *ahas11/ahas11* near isogenic lines (NILs, R20 and R54, respectively). CLPlus NILs were obtained as a result of a conversion process of susceptible wild type parental lines to their Clearfield Plus counterparts. These heterozygous lines were sown under field conditions and treated with herbicide as described above. During flowering, pollen production and viability of the ovules were assessed. Viability of the ovules was assessed by crossing each male sterile induced plant with a fertile susceptible line, determining the production of seeds and relating it with the number of seeds produced by the same untreated line under open pollinated conditions. (N=20). To determine if there exists gametophytic selection, seeds obtained from these crosses were planted under greenhouse conditions and sprayed at V2-V4 with 40g a.i. ha<sup>-1</sup> imazapyr to determine the percentage of tolerant plants.

## RESULTS

Susceptible lines were killed by all herbicide treatments. Homozygous Imisun lines treated with 40 or 80 g a.i. ha<sup>-1</sup> at V8 showed heads with a normal morphology but they were male and female sterile. The same treatment applied at R1 resulted in several plants with deformations of the head, as well as male and female sterility (Table 1). Heterozygous Imisun lines showed the same abnormal development of the capitulum but at lower doses of herbicide application than their homozygote isolines, and complete male and female sterility. Homozygous CLPlus lines showed no phytotoxicity symptoms and were male and female fertile at both herbicide doses applied at V8 or R1. Heterozygous Clearfield Plus lines, on the other hand, did not show any deformation of the head at any of the tested doses or development stage of application. Nevertheless, they were consistently male sterile after herbicide application. Under open pollination conditions, both homozygous and heterozygous CLPlus lines produced seeds (Table 1).

**Table 1.** Phenotypic characterization of five near isogenic lines carrying different *Ahas11* allele combinations after 80 g a.i. h<sup>-1</sup> imazapyr treatments during late vegetative (V8) and early reproductive (R1) stages.

Line †	Ahas11	V8			R1		
		HM <sup>1</sup>	P <sup>2</sup>	SP <sup>3</sup>	HM	P	SP
B8 CL Plus	<i>Ahas11-3/Ahas11-3</i>	N	N	N	N/AN	N	N
B8 Imisun	<i>Ahas11-1/Ahas11-1</i>	N	MS	S	N	MS	S
B8 Susceptible	<i>ahas11/ahas11</i>	-*	-	-	-	-	-
B8 Susc / B8 CL Plus	<i>ahas11/Ahas11-3</i>	N	MS	N	N	MS	N
B8 Susc / B8 Imisun	<i>ahas11/Ahas11-1</i>	N/AN	MS	S	AN	MS	S

<sup>1</sup> HM = Head morphology. N = normal morphology; AM = abnormal morphology

<sup>2</sup> P = Pollen production. N = normal pollen production and normal morphology of pollen grains; MS = male sterile

<sup>3</sup> SP = Seed production. N = normal seed production; S = sterile, the head did not produce any seed or seeds are empty

\* - All plants were killed by the herbicide treatment

† = Results are given for the B8 inbred line and its NILs. Results for the B7 and B9 inbred lines were exactly the same and are not shown.

To further characterize the male sterility observed in heterozygous CLPlus genotypes after imidazolinone application in V8 or R1, we used two restorer lines (R20 and R54) and the same treatments described above. Again, all the heterozygous plants treated at V8 or R1 with 40 or 80 g a.i. ha<sup>-1</sup> of imazapyr were male-sterile, whereas the CLPlus homozygous counterparts of both restorer lines used as checks were completely fertile. Using ten herbicide-induced male sterile plants per herbicide treatment, we analyzed their seed production and the segregation pattern for tolerance in the progeny obtained by crossing them with pollen of an herbicide susceptible inbred line. Average production of seeds, as percentage of the untreated checks, was 54.2±6.1% and 471.1±11.3% for the inbred line R54 treated at R1 and V8 respectively, and 45.2 ± 6.3% and 58.2 ± 9.1% for the inbred line R20 treated at R1 and V8, respectively.

The obtained seed from these crosses were planted under greenhouse conditions and sprayed at V2-V4 with 40 g a.i. ha<sup>-1</sup> to determine the tolerance: susceptibility ratio in the progeny. The expected tolerance: susceptibility ratio in these BC1F1 populations should be 1:1, but in all cases the observed ratio deviated significantly from the expected ratio. In fact, in almost all cases it was not observed any segregation,

since all the individuals in the progeny were tolerant. In some cases, some susceptible progeny was observed; especially when the lower herbicide dose was used (Tables 2 and 3).

**Table 2.** Herbicide tolerance analysis of the progeny obtained from induced male sterile CLPlus heterozygous lines treated with 40 and 80 g a.i ha<sup>-1</sup> of imazapyr during V8 and pollinated with a susceptible (*ahas11*) line. T: tolerant; S: susceptible.

Cross #	R20/R720 CL Plus				R54/R754 CL Plus			
	40 g a.i. ha <sup>-1</sup>		80 g a.i. ha <sup>-1</sup>		40 g a.i. ha <sup>-1</sup>		80 g a.i. ha <sup>-1</sup>	
	T	S	T	S	T	S	T	S
1	96	3	78	0	84	0	96	0
2	90	0	92	0	79	2	92	0
3	72	0	80	1	123	4	87	0
4	140	2	84	0	142	0	100	1
5	125	1	108	0	112	5	122	0
6	150	2	120	0	128	0	114	0
7	94	5	132	2	132	2	147	2
8	98	2	94	0	74	3	138	0
9	104	0	72	1	80	0	124	3
10	112	4	101	0	94	0	79	0

**Table 3.** Herbicide tolerance analysis of the progeny obtained from induced male sterile CLPlus heterozygous lines treated with 40 and 80 g a.i ha<sup>-1</sup> of imazapyr during R1 and pollinated with a susceptible (*ahas11*) line. T: tolerant; S: susceptible

Cross #	R20/R720 CL Plus				R54/R754 CL Plus			
	40 g a.i. ha <sup>-1</sup>		80 g a.i. ha <sup>-1</sup>		40 g a.i. ha <sup>-1</sup>		80 g a.i. ha <sup>-1</sup>	
	T	S	T	S	T	S	T	S
1	87	0	95	0	112	0	145	0
2	92	1	86	0	104	7	132	0
3	78	0	103	1	84	0	109	0
4	123	2	114	0	105	3	98	2
5	154	2	128	2	98	0	98	1
6	105	0	146	0	96	4	108	0
7	94	3	132	0	104	2	84	0
8	96	7	102	2	114	1	78	1
9	102	4	94	0	117	0	142	0
10	114	0	83	3	102	0	103	0

## DISCUSSION

The results obtained are consistent with previous observations regarding differences in tolerance to imidazolinone applications between CLPlus and Imisun genotypes (Sala et al., 2008a & b, 2011). However, these results also highlight the organ specific pattern of gene expression of the sunflower *Ahas1* genes. In fact, herbicide applications during late vegetative or early reproductive stages of development are well tolerated by heterozygous CLPlus plants, but these treatments result in a failure of the anthers to fully develop, rendering the plants male sterile. It appears that this failure is the result of a decrease in the expression level of the *Ahas11* gene in the anthers which determines that there are not enough tolerant targets in these tissues to complete their normal development. This divergent pattern of expression of different members of the AHAS multigene family is also observed in other paleopolyploid species (*Brassica napus*, Ouellet et al., 1992; *Gossypium hirsutum*, Grula, 1995) like sunflower.

As was also observed in other HT crops (i.e.: glyphosate resistant soybean, Walker et al., 2006) due to gametophytic selection after herbicide applications in late vegetative stages, instead of a normal 1:1 ratio of tolerant to susceptible ovules, a selection of female gametes carrying resistant alleles occurred.

These results allowed us to develop a system of male-sterilization of restorer testers. This new system involves the following steps: (i) to obtain heterozygous *Ahas11-3* (CLPlus) inbred lines; (ii) to male sterilize plants of these lines by spraying them at V8-R1 using an imidazolinone herbicide, and (iii) to multiply the system by crossing the male-sterilized heads with the susceptible restorer line.

The method described in this work allows breeders to test the combining ability of maintainer lines in early stages of development. Previously this was done by backcrossing to a sterile CMS source, which is

laborious and time consuming. This new method can be used with any type of germplasm. It confers greater flexibility in application timing (compared to gibberellic acid treatments), allows the testing of fertile hybrids (a main difference to the system that relies on CMS testers) and permits a rapid change of the genetic background of the tester (a difference with the systems based on nuclear male sterility). Moreover, it can be used to develop any type of recurrent selection method in the breeding program, and increase the frequency of herbicide tolerant plants by gametophytic selection.

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