Development of CLHA-Plus: a novel herbicide tolerance trait in sunflower conferring superior imidazolinone tolerance and ease of breeding

Carlos Sala[1], Mariano Bulos[1], Mariel Echarte[1], Sherry Whitt[2], Gregory Budziszewski[2], William Howie[2], Bijay Singh[2], Brigitte Weston[2]

[1]Nidera S.A., Departamento de Biotecnología, Casilla de Correo 6, 2600 Venado Tuerto, Santa Fe, Argentina, csala@nidera.com.ar

[2]BASF Plant Science L.L.C., 26 Davis Drive, Research Triangle Park NC 27709, USA. brigitte.weston@basf.com

ABSTRACT

A novel imidazolinone (IMI)-tolerance trait, CLHA-Plus, was developed through EMS mutagenesis and subsequent selection with imazapyr. The objective of this work was to determine the relative IMI tolerance level of this new mutation with respect to the current commercial IMISUN, by: (a) challenging genetic materials containing the new mutation and/or the old IMISUN with different doses of imazamox and imazapyr under a range of different environmental field conditions and (b) testing the *in vitro* acetohydroxyacid synthase (AHAS) activity of the CLHA-Plus and IMISUN hybrids at increasing levels of imidazolinone herbicides. Lines and hybrids homozygous for the CLHA-Plus mutation demonstrated better tolerance to imidazolinone herbicides than commercially available IMISUN sunflowers which are homozygous for the already known resistant gene (*Imr1*) and an uncharacterized modifier/enhancing factor (*Imr2*). Hybrids heterozygous for the combined mutations CLHA-Plus/IMISUN demonstrated similar field tolerance levels as well as similar AHAS enzyme IMI dose responses to hybrids homozygous for the novel CLHA-Plus mutation. Thus, a higher level of tolerance to imidazolinones can be achieved by allelic substitution of IMISUN by CLHA-Plus in only one of the parental lines of a CLEARFIELD® hybrid, which –in turn- permits a more rapid deployment of this new allele in the hybrid sunflower crop.

Key words: acetohydroxyacid synthase (AHAS) mutation – acetolactate synthase (ALS) mutation sulfonylurea – CLEARFIELD sulfonylurea – CLHA-Plus sulfonylurea – herbicide tolerance sulfonylurea – imidazolinone tolerance.

INTRODUCTION

The imidazolinone family of herbicides control weeds by inhibiting a key enzyme in the branched chain amino acid biosynthetic pathway, acetohydroxyacid synthase (AHAS) or acetolactate synthase (ALS) (Shaner et al., 1984; Tan et al., 2004). Imidazolinones, such as imazapic, imazethapyr, imazapyr and imazamox, are key herbicide components in the CLEARFIELD® production system, which provides effective and extended weed control when used in combination with elite non-GM, imidazolinone tolerant, seed varieties. The CLEARFIELD production system is used commercially in North America, Europe, South America, Asia, Australia and Africa in combination with the following crops: canola (oilseed rape), maize (corn), lentils, rice, wheat, and sunflowers (Pfenning et al. 2008).

The development of CLEARFIELD sunflowers started in 1996, when imidazolinone-tolerant (Pursuit®) wild sunflowers were discovered in a field in Kansas, USA. Seed of these plants were sent to the USDA in Fargo (North Dakota, USA) for subsequent crossing to cultivated sunflower lines (Al-Khatib et al., 1998)

The commercial imidazolinone tolerance trait, IMISUN, which arose from this original USDA introgression work, was commercially launched in the USA, Argentina and Turkey in 2004. From its initial launch up to the present, the IMISUN trait has seen growth in both the number of countries that have adopted this technology and in market share. Sunflower hybrid varieties are currently being commercialized under the CLEARFIELD trademark in 15 major sunflower growing countries in the European Union (EU), Eastern EU, North America (NA), and South America (SA).

The inheritance of IMISUN appears to be additively controlled by two genes, where one (*Imr1*) is a partially dominant gene and the other (*Imr2*) is a modifier or enhancer gene/factor (Miller and Al-Khatib, 2002; Bruniard and Miller, 2001). To produce IMISUN sunflower lines that express commercial tolerance levels to imidazolinone herbicides, both factors need to be homozygous in the final variety (*Imr1Imr1/Imr2Imr2*). Since there are no diagnostic methods available for detecting the presence of the

® CLEARFIELD and the unique CLEARFIELD symbol are registered trademarks of BASF

modifier/enhancer factor *Imr2*, all breeding selections rely on the phenotypic evaluation of the plants that have been sprayed with the imidazolinone herbicide. This phenotypic selection process can be qualitative, depending on the segregation of the IMISUN genes, making the selection of homozygote lines often tedious and time consuming.

In an effort to develop a herbicide tolerance trait which does not require a modifier/enhancing factor, Nidera S.A. together with BASF initiated a seed mutagenesis program in sunflowers to discover new AHAS mutations that would simplify the breeding process. As a result, a new tolerance trait named CLHA-Plus was discovered (Sala et al., 2008). The objective of this work is twofold: (a) to determine the relative imidazolinone tolerance level of this new herbicide tolerance mutation with respect to the current commercial IMISUN and (b) to test the *in vitro* AHAS activity of the CLHA-Plus and IMISUN hybrids at increasing levels of herbicides.

MATERIALS AND METHODS

A sunflower line, BTK47, specifically selected for lack of an E-factor (*imr1 imr1 / imr2 imr2*), was subjected to EMS seed mutagenesis (Sala et al., 2008). An $M_{2:4}$ line which survived imazapyr field selection, was selected for subsequent crossing and enzyme activity studies. This line was named GM40.

Field Evaluation of the CLHA-Plus Trait

The CLHA-Plus mutant allele was introgressed into different maintainer, restorer and sterile inbred lines. Homozygous CLHA-Plus inbreds were crossed with either WT inbreds (containing no herbicide tolerance mutation), homozygous CLHA-Plus inbreds, or homozygous IMISUN inbreds to produce different F_1 mutant allele zygosity combinations (Table 1). These entries, along with several regionally adapted CLEARFIELD® IMISUN commercial variety checks, were field tested for imidazolinone tolerance at numerous locations in North America, South America and EU from 2005 to 2008 (Table 2).

Table 1. Entry list for herbicide tolerance field evaluations (2007)

Entry	Line Description	AHASL1 Allele Zygosity
1	GM40	CLHA-PLUS Homozygous
2	cmsGM40 x R733	CLHA-PLUS Homozygous
3	cmsBTK47 x R731	CLHA-PLUS Heterozygous
4	IA9 x R733	IMISUN / CLHA-PLUS Heterozygous
5	IA9 x RHA426	IMISUN Homozygous
6	B7imi (IMISUN1)	IMISUN Homozygous
7	cmsB7 x RHA426	IMISUN Heterozygous
8	B7	WT

Table 2. Location list for herbicide tolerance field evaluations (2005 - 2007)

Year	Country	Nearest Town Location, State or Province
2005	USA	Velva, North Dakota
2005/2006	Argentina (AR)	Venado Tuerto, Santa Fe
2006	USA	Velva, North Dakota
20062007	Argentina	Venado Tuerto, Santa Fe
20062007	Argentina	Balcarce, Buenos Aires
2007	Argentina	Laguna Blanca, Formosa
2007	USA	Velva, North Dakota
2007	USA	Hickson , North Dakota
2007	France (FR)	Angers
2007	France	Saintes
2007/2008	Argentina	Venado Tuerto, Santa Fe
2007/2008	Argentina	San Jeronimo, Santa Fe
2007/2008	Argentina	Balcarce, Buenos Aires

Table 3. Imidazolinone treatment list for herbicide tolerance field evaluations (2007)

Treatment Number	Herbicide Treatment	Herbicide Product Formulation
1	Untreated	
2	50 g ai/ha imazamox + 0.25% (v/v) NIS*	Beyond 120 g/l LC
3	100 g ai/ha imazamox + 0.25% (v/v) NIS*	Beyond 120 g/l LC
4	200 g ai/ha imazamox + 0.25% (v/v) NIS*	Beyond 120 g/l LC
5	160 g ai/ha imazapyr + 0.25% (v/v) NIS*	Arsenal 240 g ai/L
6	320 g ai/ha imazapyr + 0.25% (v/v) NIS*	Arsenal 240 g ai/L

*NIS = non-ionic surfactant = Induce 90SC (90%)

The entries at each location in 2007 and 2007/2008 were arranged in a randomized two factorial split plot design consisting of 3 replications for each treatment combination. Factor A was the herbicide treatment (Table 3), and factor B was the sunflower entry (Table 1). The plot size was 2 rows x 7 m and the seeding rate was consistent with local agronomic practices. The herbicide treatment was applied at the 2-4 leaf stage with a tractor mounted boom (20 gallons/acre or 200 litres/ha). Treatment 2 was only applied at 2 locations in France.

Crop injury (% phytotoxicity) ratings were evaluated at 6 - 10 days after treatment and at 16 - 21 days after treatment. Percent phytotoxicity was recorded as the average amount of plant damage in a given plot, where a rating of '0%' indicated no damage to plants relative to the untreated plot. A rating of 10% to 40% indicated increasing levels of chlorosis (where 40 would be complete yellowing of the leaves). A rating of 50% or higher indicated that the plants demonstrated complete yellowing as well as increasing levels of leaf necrosis. A rating of '100%' indicated complete necrosis (death) of the plants.

The emergence, days to flower, days to end of flower and maturity were also assessed for each plot at each location (data not shown). The data were subjected to an ANOVA analysis.

Enzyme Assay for AHAS Activity

Twelve greenhouse grown sunflower plants from each of the lines depicted in Table 4 were bulked and subjected to an AHAS enzyme activity assay (Singh et al., 1988). Each activity assay was repeated twice. Due to the large number of samples, the experiment was split into two sets (Table 4).

Table 4. Line descriptions and corresponding AHASL1 mutation allele zygosities

Set	Line Description	AHASL1 Allele Zygosity
1	cmsGM40 x R733	CLHA-PLUS Homozygous
1	IA9 x R733	IMISUN/CLHA-PLUS Heterozygous
1	IA9 x RHA426	IMISUN Homozygous
1	B7	WT
2	GM40	CLHA-PLUS Homozygous
2	cmsBTK47 x R731	CLHA-PLUS Heterozygous
2	B7imi (IMISUN1)	IMISUN Homozygous
2	cmsB7 x RHA426	IMISUN Heterozygous
2	B7	WT

Protein extracts from young, actively growing leaves from four week old plantlets were prepared and subjected to an AHAS inhibition assay (Singh et al., 1988). Assays were conducted in a 96-well format. Fifty µl of inhibitor was added to each well containing 50 µl of soluble protein extract to give final concentrations of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µM imazamox or 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µM imazapyr. Zero herbicide controls were also included for each line. Reactions were processed as outlined by Singh et al. (1988). Absorbance was measured at 530 nm. AHAS activity, expressed as the mean of the absorbance values for each treatment, was presented as a percentage of the mean of the zero-herbicide controls.

RESULTS AND DISCUSSION

To assess HT genes for their relative tolerance level, two approaches were used. The first approach measured herbicide injury in the field under a range of environmental stringencies (locations and years in combination with different herbicide doses), and the second approach tested the target enzyme (*in vitro*) with increasing levels of herbicide.

In the field, the crop injury phenotype can be attributed to the interaction between genotype and environment (GxE). The genotypic factor in a herbicide tolerant (HT) plant is the sum of the HT gene(s) plus the remaining genetic background, and the interaction between the two. The environmental component (E) is a sum of abiotic (i.e. weather, soil) and biotic factors (i.e. insect, disease and weed pressure) coupled with the effect of the herbicide dose. An example of this environmental effect is seen in Fig. 1, where a variation in phytotoxicity of the same genotype grown in four different locations (Velva ND, Angers FR, Saintes FR, Formosa AR) at the same dose rate (200 g ai/ha imazamox) is observed. To better understand the environmental factor associated with this trait, we calculated the mean phytotoxicity index (PI) of the current commercial, regionally adapted, IMISUN checks at 6 – 10 days after herbicide treatment across many locations over 3 years. PI values for different hybrids carrying the CLHA-Plus mutation were plotted against the mean PI values of the IMISUN checks to evaluate the relative resistance level of the new mutation across a range of Es (Fig. 2). As can be seen in the x axis of Fig. 2, the combination of locations with herbicide doses produced a diverse array of Es, which ranged in PI mean values from 5.9 to 78 for the imazamox treatments (not shown); and 2 to 100 for the imazapyr treatments (Fig. 2). The y=x line represented the mean PI value for the IMISUN checks across all Es.

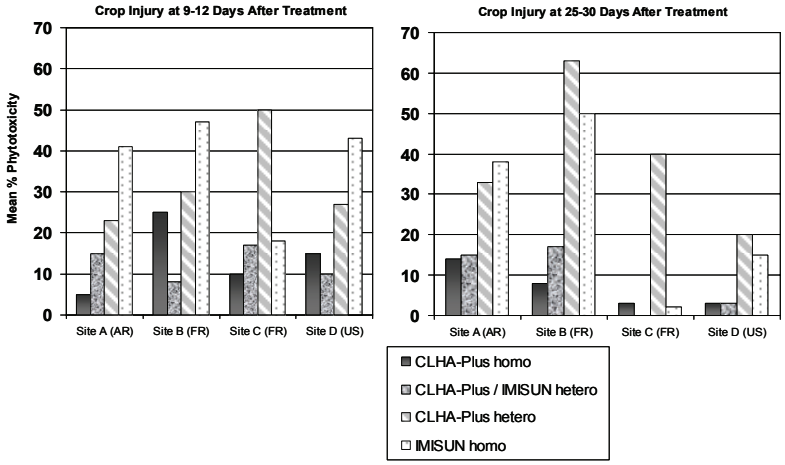

Fig. 1. Crop Injury (Mean % Phytotoxicity) at 200 g ai/ha Imazamox at 4 Field Locations in 2007 for 4 Different Types of Hybrids (see legend)

The results obtained following imazamox treatments are described in the following paragraph. The CLHA-Plus homozygous hybrids showed an increase in PI as the environmental component became more severe. However, the slope of the regression line (b= 0.149± 0.0667, P<0.0375) indicated that the level of crop injury, as a function of environmental stringency, increased at a lower rate than the IMISUN checks. Hybrids which were heterozygous for the double CLHA-Plus / IMISUN stack showed a similar response to environmental stringency (b=0.39±0.05, P<0.0001) as the homozygous CLHA-Plus hybrids. On the other hand, hybrids containing the CLHA-Plus mutation in a heterozygous state (CLHA-Plus/WT) demonstrated higher crop injury ratings than the IMISUN checks at lower levels of environmental stringency, as shown by the higher y-intercept value of the regression line (a=15.3±2.67). When the severity of the environmental component was increased, these CLHA-Plus heterozygous hybrids showed a better performance than the IMISUN checks, as was shown by the slope of its linear equation (b=0.45±0.062, P<0.0001). The same was observed in Fig. 2 when the same entries, in the same environments, were challenged with imazapyr (environmental stringencies for each genotype are summarized by the regressions in the Fig. 2 Legend).

To substantiate the herbicide tolerance effect observed in the field, the same herbicide tolerance gene combinations were subjected to AHAS enzyme inhibition studies. These studies were conducted on the bulk of 12 individuals from each entry in Table 1. The mean of two replications are represented in Fig. 3 for Set 1 (Table 1) and in Fig. 4 for Set 2 (Table 1). An untreated control sample was included to provide a baseline for 100% AHAS enzyme activity. The AHAS activity in the CLHA-Plus homozygous hybrid

treated with 100 µM imazamox was 69% of the untreated control, and for the 100 µM imazapyr it was 64% of the untreated control (Fig. 3). The activity of the AHAS enzyme in the CLHA-Plus/IMISUN heterozygous hybrid was 59% and 60% for extracts treated with 100 µM imazamox and 100 µM imazapyr respectively (Fig. 3). The IMISUN homozygous hybrid line, current commercial CLEARFIELD® product, demonstrated AHAS activities of 36% of untreated control and 42% of untreated control at 100 µM imazamox and 100 µM imazapyr respectively (Fig. 3), which is lower than the activities of both the CLHA-Plus homozygous hybrid and the CLHA-Plus/IMISUN heterozygous hybrid.

In the second set of data, the IMISUN homozygous hybrid (current commercial CLEARFIELD product) performed similarly to the CLHA-Plus heterozygous hybrid (Fig. 4). More specifically, the IMISUN hybrid demonstrated 26% activity at 100 µM imazamox and the CLHA-Plus heterozygous hybrid had 30% activity at 100 µM imazamox. In contrast, the AHAS enzyme extract from the CLHA-Plus homozygous hybrid demonstrated the least amount of inhibition with increasing levels of imazamox, demonstrating activities of 63% and 60%, relative to the untreated control, at 50 µM and 100 µM imazamox respectively (Fig. 4). The WT line (B7) was genotypically identical in both experimental sets and demonstrated a variance of 6% activity at the 100 µM imazamox level between the two experiments (17% AHAS activity relative to the untreated control in Set 1 (Fig. 3) and 11% AHAS activity relative to the untreated control in Set 2 (Fig. 4).

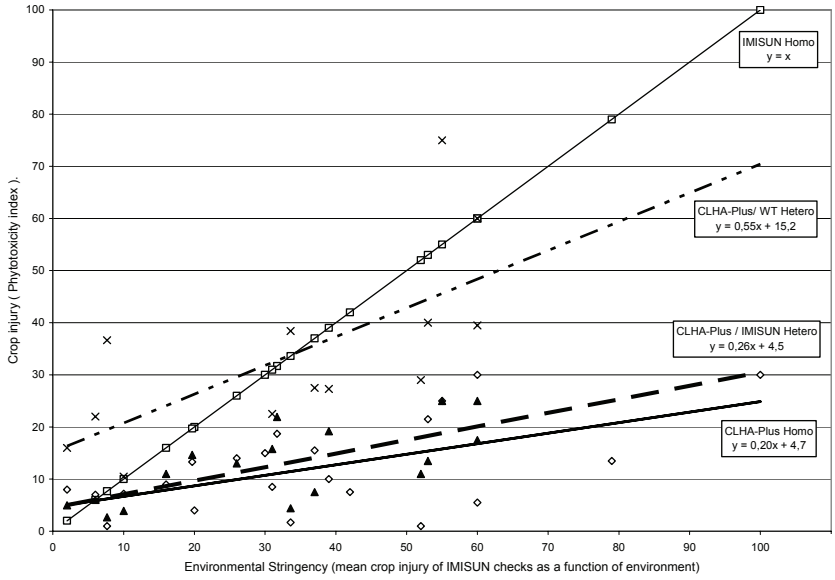

Fig. 2. Crop Injury of different types of sunflower hybrids carrying the CLHA-Plus mutation after imazapyr application ((CLHA-Plus homozygous: b= 0.20 ± 0.06, P<0.048 CLHA-Plus /IMISUN heterozygous: b: 0.26 ± 0.07, P<0.0019; CLHA-Plus /WT: b: 0.55±0.18, P< 0.0109)

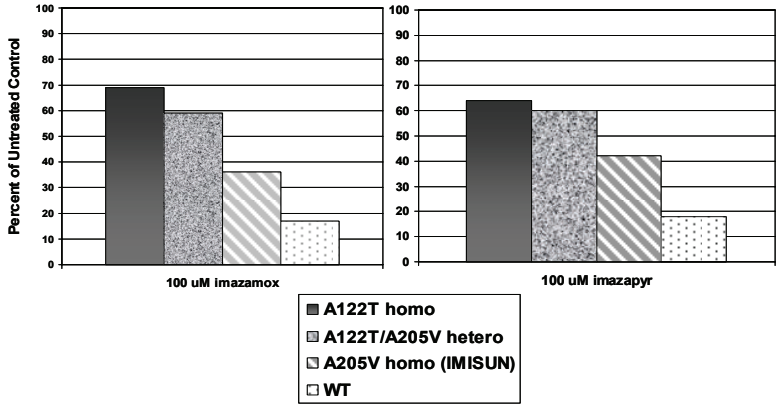

Fig. 3. AHAS Enzyme Activity (as Percent of untreated controls) of Four Sunflower Lines at 100 micromoles of Imazamox and 100 micromoles of Imazapyr

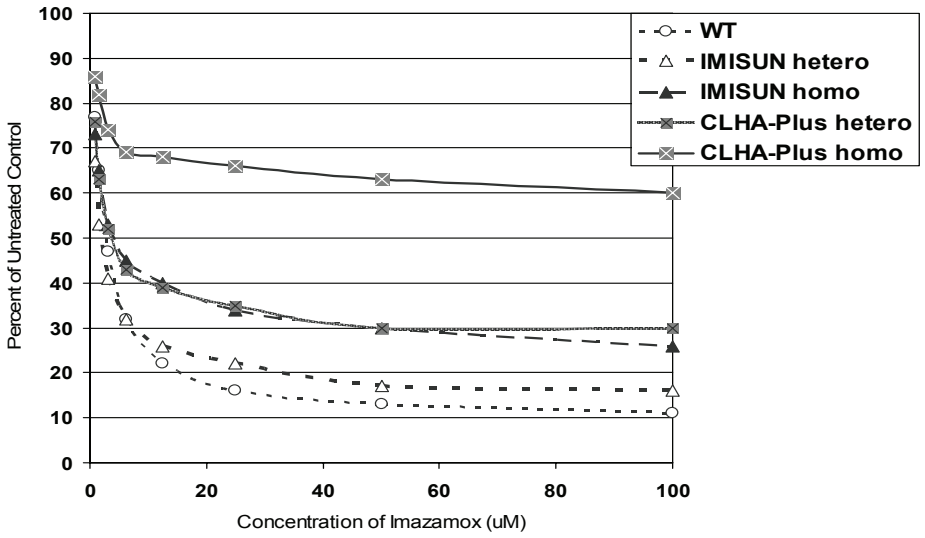

Fig. 4. AHAS Enzyme Activity (Percent of untreated controls) of Five Sunflower Lines with Increasing Levels of Imazamox

Based on field and AHAS enzyme activity data, it was determined that the novel CLHA-Plus mutation provides superior herbicide tolerance to imidazolinones versus the current IMISUN mutation. Commercial levels of herbicide resistance in IMISUN sunflowers require the combination of two genetic factors in a homozygous state due to the moderate level of resistance conferred by *Imr1*. In contrast, by using the CLHA-Plus mutation alone, the *Imr2* enhancer is no longer necessary to achieve commercial levels of tolerance. Most importantly, the results demonstrate that CLHA-Plus can be used either as a homozygous single gene HT trait or as a heterozygous stack together with the IMISUN HT trait, providing enhanced levels of tolerance, greater flexibility in weed control and facilitating the deployment of this new mutation in the CLEARFIELD® Production System.

ACKNOWLEDGEMENTS

The authors would like to thank the following employees at Nidera S.A.: Guillermo San Román, Leandro Amela, Juan Pablo Bizzera and Néstor Duarte and at BASF: Thierry Guillet, Patrice Cabiac, Daniel Rolon, Vince Ulstad and Julie Roland for their expert advice and assistance with this project.

REFERENCES

Al-Khatib, K., J.R. Baumgartner, D.E. Peterson, and R.S. Currie. 1998. Imazethapyr resistance in common sunflower (*Helianthus annuus*). Weed Sci. 46:403-407.

Bruniard, J.M., and J.F. Miller. 2001. Inheritance of imidazolinone herbicide resistance in sunflower. Helia 24:11-16.

Miller, J.F., and K. Al-Khatib. 2002. Registration of imidazolinone herbicide-resistant sunflower maintainer (HA425) and fertility restorer (RHA426 and RHA427) germplasms. Crop Sci. 42:988-989.

Pfenning, M., G. Palfay, and T. Guillet. 2008. The CLEARFIELD® technology – A new broad-spectrum post-emergence weed control system for European sunflower growers. J. Plant Dis. Prot. (submitted).

Sala, C.A., M. Bulos, and A.M. Echarte. 2008. Genetic analysis of an induced mutation conferring imidazolinone resistance in sunflower. Crop Sci. (accepted).

Shaner, D.L., P.C. Anderson, and M.A. Stidham. 1984. Imidazolinones: Potent inhibitors of acetohydroxyacid synthase. Plant Physiol. 76:545-546.

Singh, B.K., M.A. Stidham, and D.L. Shaner. 1988. Assay of acetohydroxyacid synthase. Ann. Biochem. 171:173-179.

Tan, S., R.R. Evans, M.L. Dahmer, B.K. Singh, and D.L. Shaner. 2005. Imidazolinone-tolerant crops: history, current status and future. Pest Manag. Sci. 61:246-257.