Clearfield® Plus Technology in Sunflowers

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- The Clearfield® production system, which is based on ImiSun, is an essential component of hybrid sunflower oil and confectionery production. Since its launch in 2003 it has been adopted in sunflower growing regions around the world due to a wider spectrum of weed control; a higher level of consistency; greater flexibility in the timing of herbicide applications; season-long weed control, and a low rate of application. With the development of the Clearfield Plus (CLPlus) production system, which is based on a single gene with higher levels of tolerance to imidazolinones, new findings and subsequently new benefits have been discovered as the trait progressed from concept, to research, to development and as of 2010 to the marketplace. It is the intention to review these findings and benefits as they relate to two key stakeholders in the sunflower seed industry pipeline, namely the variety developer or breeder and the grower.
- Originally, the CLPlus trait was developed with the goal to select a more tolerant imidazolinone gene in sunflower based on a single gene. In 2000, a research and development program was initiated with Nidera S.A. to realize that goal. By 2006, an improved trait, developed through seed mutagenesis of an elite cultivated sunflower line, was confirmed. This novel trait, based on a new mutation in the *Ahasl* gene, called *CLHA-Plus* or *Ahasl1-3*, was characterized at the molecular level, at the whole plant level and at the agronomic level. It was during this characterization phase that many of the new benefits, such as improved oil content, improved stability and reliability of the herbicide tolerance and improved yield through better weed management were discovered. More recently, and following the creation of a limited number of isogenic hybrids with the different technologies, a correlation between CLPlus and higher yield and higher grain oil content was observed relative to the ImiSun trait
- Much of what we have learned to date concerning the CLPlus trait is based on germplasm from Nidera. Even though many different lines and hybrids have been tested in South America, North America and Europe the fundamental question still remains as to how this trait will function in other breeders' programs. To assist our primary stakeholder, the breeders, to rapidly introduce CLPlus into their breeding programs and future varieties, a number of tools have been developed to help select, breed, assure quality and qualify future hybrids. Similarly for the grower, a number of CLPlus tools and options are under development to improve the flexibility and reliability of the weed control system as part of a longer term viable product stewardship effort

Helianthus annuus; imidazolinone tolerance; *Ahasl;* Clearfield Plus ; ImiSun; *CLHA-Plus*

INTRODUCTION

In 2003, in cooperation with leading global and regional seed partners, BASF introduced sunflowers into the growing family of successful Clearfield® Production System crops that included canola/oilseed rape, maize, rice and wheat. Within a few years, the Clearfield (CL) Production System for sunflower had become known as an essential component in hybrid sunflower oil and confectionary production. The original CL trait in sunflowers, ImiSun, is based on a natural acetohydroxyacid synthase (AHAS) mutation discovered in 1996 in a wild sunflower population growing in a soybean field in the United States (Al-Khatib et al, 1998). This trait was bred into cultivated sunflowers and released to the breeding community in USDA inbred lines (Miller and Al-Khatib, 2002, Jocic, 2004). In 2000, a research and development program was initiated with Nidera S.A. to deliver a more efficient breeding system based on a single gene, and sunflowers with greater crop tolerance to imidazolinones. By 2006, BASF confirmed an improved trait, which was developed through seed mutagenesis in an elite cultivated sunflower line, and named it Clearfield Plus (CLPlus). This novel trait was based on a new mutation in the *Ahasl* gene, called *CLHA-Plus* or *Ahasl1-3* (Sala et al., 2008a, Sala et al., 2008b). Four years later, BASF received PNT (Plant Novel Trait) regulatory approval in Canada for Clearfield Plus (*CLHA-Plus*) (Canadian Food Inspection Agency, Approved PNTS), giving producers and exporters globally the freedom to sell their grain and grain products into distribution channels that could end in Canada. Like ImiSun, the CLPlus trait is a non-GMO trait.

TECHNICAL CONSIDERATIONS

CLPlus is based on a single gene, the *CLHA-Plus* or *Ahasl1-3* gene. Detailed segregation and molecular studies confirmed that the *Ahasl1-3* gene resides on the same gene as the ImiSun target site imidazolinone tolerance gene, *Ahasl1-1*(Sala et al, 2008b). For this reason, these two mutations can never both exist in a homozygous state in one sunflower individual. Initial greenhouse and field studies on both inbreds and hybrids showed that when the *CLHA-Plus* or *Ahasl1-3* gene is in a homozygous state or in a heterozygous *Ahasl1-1/Ahasl1-3* combination, a higher than commercial imidazolinone tolerance level was observed than in ImiSun (*Ahasl1-1/Ahasl1-1*) inbreds and hybrids (Sala et al., 2008a). This was confirmed by aggregating phytotoxicity or crop injury data from ImiSun (*Ahasl1-1/Ahasl1-1*), CLPlus Hetero (*Ahasl1-1/Ahasl1-3*) and CLPlus Homo (*Ahasl1-3/Ahasl1-3*) inbreds and hybrids field tested in uniform trial designs at 16 sites over 6 years and 9 locations in Argentina, USA, Hungary and France. Different rates of imazamox and imazapyr ranging up to 6x the regular field dose rate (referred to as 'Product Category' in Figures 1 through 3) were tested. ImiSun local checks were used for each region across years and locations. A total number of 1887 crop phytotoxicity observations were analyzed using Tukey's Studentized Range (HSD) Test. Each observation was assessed between 8 and 14 days after herbicide application. The outcome of this analysis graphs the mean percentage crop phytotoxicity along with the variance as a function of imidazolinone product and rate for each different *Ahasl1* genetic category (Figures 1, 2 and 3). Besides demonstrating a lower % phytotoxicity, CLPlus Hetero and CLPlus Homo entries exhibited a much smaller range in variation (Figures 2 and 3) across diverse environments when compared to the ImiSun entries (Figure 1).

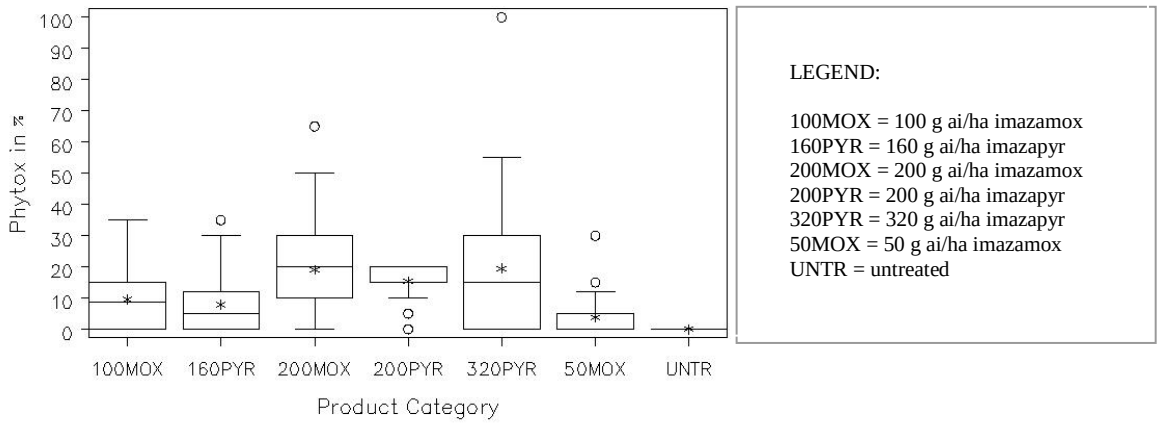

Figure 1: Mean % Phytotoxicity of ImiSun Hybrids Treated with Different Imidazolinone Products and Rates

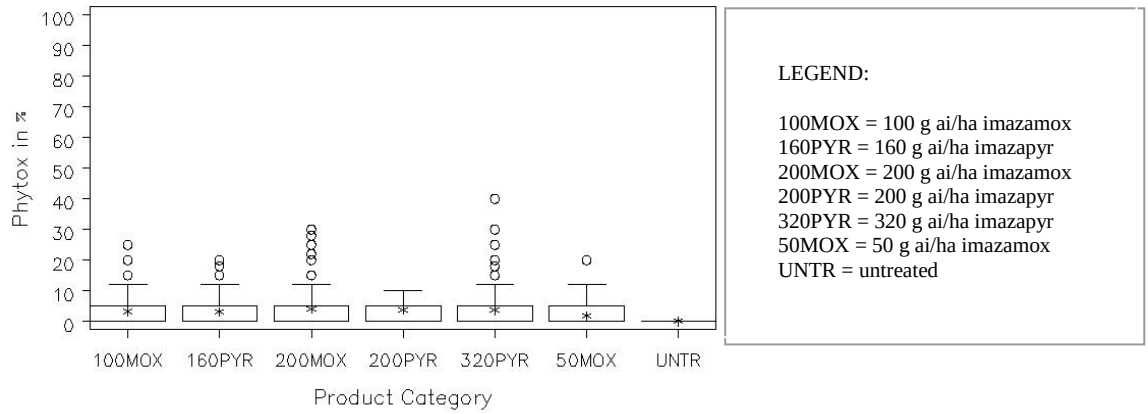

Figure 2: Mean % Phytotoxicity of CLPlus Hetero Hybrids Treated with Different Imidazolinone Products and Rates

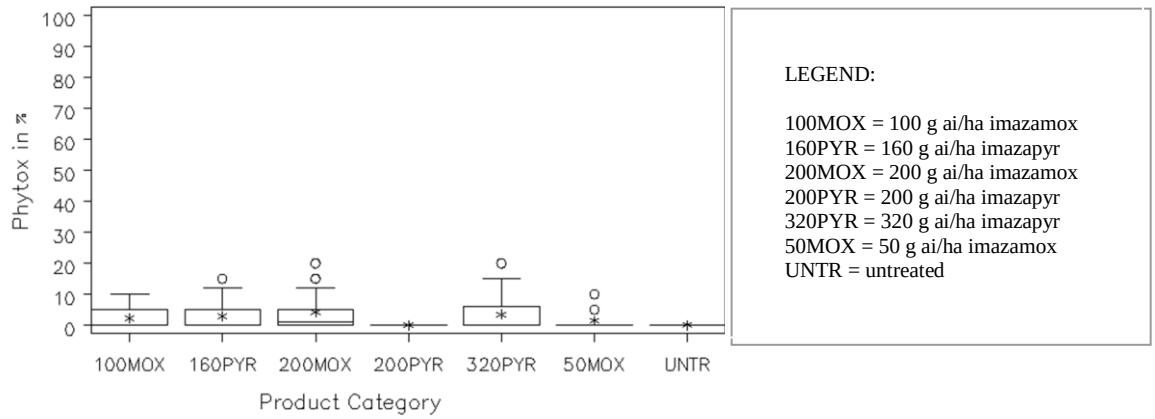

Figure 3: Mean % Phytotoxicity of CLPlus Homo Hybrids Treated with Different Imidazolinone Products and Rates

These findings are supported by a larger aggregate study conducted in 2011 by Sala et al (2012b) which assessed ImiSun and CLPlus hybrids for their relative tolerance level at more locations over the same 6 year period. The authors conducted a regression analysis of the mean phytotoxicity for each hybrid and herbicide dose as a function of the mean phytotoxicity of current commercial, regionally adapted Imisun checks, and used the latter as an environmental index. They concluded that CLPlus hybrids had a superior level of imidazolinone tolerance compared to ImiSun hybrids. CLPlus hybrids also provided better tolerance stability and reliability allowing them to better cope with unpredictable environmental variation than their ImiSun counterparts.

BREEDING WITH CLPLUS

The inheritance of the ImiSun trait is controlled by at least two or more genes; one gene is the target site AHAS mutation (*Ahasl1-1*) and the others are enhancers (Bruniard and Miller, 2001) or non-target site tolerance factors (Sala et al., 2012a). There are no diagnostic methods yet available to detect the presence of the non-target site factors, therefore breeding selections rely on phenotypic evaluations of plants that have been sprayed with Clearfield herbicides. The phenotypic selection process is tedious and time-consuming and due to the segregation of multiple genes and factors, requires the screening of large numbers of plants and lines. Each generation poses a risk for the loss of a non-target site factor as the breeder advances the materials through greenhouse and nursery selections.

The *CLHA-Plus* mutation was developed by seed mutagenesis of an elite Nidera inbred line, BTK47 (Sala et al, 2008c), which lacked some if not all of the enhancing or non-target factors, as verified by this elite inbred's inability to produce an ImiSun parent with sufficient commercial tolerance. Following trait selection and characterization (Sala et al., 2008a), the CLPlus trait was backcrossed into different genetic backgrounds and distributed to seed partners as CMS and R line inbreds. The first CLPlus hybrid, Paraiso 1000 CL Plus from Nidera, was launched in 2010 in Argentina with other hybrids from other companies following in 2012 and 2013.

Two different hybrid trait formats fall under the Clearfield Plus brand. The first is a CLPlus Homo hybrid where both parents carry a homozygous copy of the *Ahasl1-3* or *CLHA-Plus gene*. The second is a CLPlus Hetero hybrid where one parent is homozygous for the ImiSun *Ahasl1-1* gene and the other parent is homozygous for the *CLHA-Plus* or *Ahasl1-3* gene. For those companies with an existing ImiSun breeding program the CLPlus Hetero hybrid option is most efficient since it uses an existing ImiSun inbred as one parent in the commercial hybrid. Upwards of one year can be saved in the conversion process if the existing ImiSun parent is a cytoplasmic male sterile (CMS) female inbred. For this reason, all first generation CLPlus commercial sunflower hybrids are CLPlus Hetero. On the other hand, preliminary data show that there may be a yield advantage to using CLPlus over ImiSun (Weston et al, 2012). If this data is corroborated in more genetic backgrounds, then it may prove worthwhile for a company to convert their female inbred to CLPlus Homo in an effort to lower commercial seed production costs through improved yields. Higher herbicide tolerance on the CLPlus Homo female side also means that better formulations with stronger adjuvants can be used to provide improved weed control (Pfenning et al, 2012) during seed production, which in turn could lead to improved female parent yield.

Breeding programs solely based on the *CLHA-Plus* gene require less resources to select high levels of herbicide tolerance in breeding lines than programs based on ImiSun. Unlike ImiSun, the *CLHA-Plus* mutation does not require additional factors (genes) (Bruniard and Miller, 2001), to achieve commercial levels of imidazolinone tolerance. The CLHA-Plus mutation was also not derived from wild sunflowers (Sala et al, 2008b), as were the ImiSun and Sures traits (Al-Khatib et al, 1998), and therefore does not carry with it wild sunflower sequences linked to the *Ahasl1* gene. These two factors free up resources in a CLHA-Plus based breeding program allowing breeders to focus more on yield and disease tolerance which will eventually lead to the production of higher yielding and better quality hybrids. To date, though, most sunflower seed breeders work with more than one herbicide tolerance system often running parallel breeding programs for ImiSun, CLPlus, and Sures (Miller and Al-Khatib, 2004). In mixed breeding programs, having molecular markers available for each herbicide tolerance trait is critical to ensuring the purity of the final hybrid product. To assist with trait selection, a variety of SNP, single nucleotide polymorphism, detection methods were developed by BASF for the detection of both the *CLHA-Plus* mutation in CLPlus as well as the *Ahasl1-1* mutation in ImiSun. These markers, along with molecular markers for identity testing, disease tolerance and improved seed composition are becoming more and more prevalent in sunflower breeding programs as unit assay costs decrease and molecular diagnostic platforms become more high-throughput.

One key success factor for a hybrid breeding program is the ability to rapidly produce and test large numbers of fertile hybrids for combining ability. CMS tester lines have limited use when combined with non-Restorer lines since the test hybrids are sterile. Also the conversion of inbreds to CMS or to the Restorer genotype is both time and labour intensive. Gibberellic acid is commonly used for chemical emasculation leading to the production of fertile test hybrids but the narrow window of application makes this approach less reliable. The CLPlus trait has a unique advantage in this regard. Lines heterozygous for the *CLHA-Plus* mutation can be effectively emasculated using a prescribed does of imazapyr at the R1 stage of sunflower development. This system provides a quick method for producing test hybrids with a wider window of application than gibberellic acid (Sala and Bulos, 2012). To date this system has been validated on many different genotypes in the Nidera breeding program. It would be important to test this system in other companies' CLPlus based breeding programs to better understand whether it can be applied across all genotypes with the same window of application, as we hypothesize.

ImiSun hybrids that qualify under the Clearfield brand must have both the *Ahasl1-1* and non-target site factors in a homozygous state to ensure commercial tolerance to imidazolinones. This tolerance is verified in regionally organized agronomic field trials where qualifying hybrids may not sustain greater crop injury than the commercial tolerance standard when treated with the specified Clearfield herbicide at the designated dose, time and method of application. Similarly, CLPlus Hetero and CLPlus Homo hybrids qualifying as Clearfield Plus must meet the same criteria. BASF is currently running a multiyear study on hybrids entering the Clearfield Plus qualification system to determine

whether molecular markers specific for the CLPlus trait can eventually replace herbicide tolerance field testing for qualification.

THE GROWER AND CLEARFIELD PLUS

In conventional sunflower fields, herbicides must be sprayed preemergence and require rainfall to become activated. Oftentimes rainfall is insufficient to fully activate residual herbicides applied preemergently to the weeds and the resulting weed control is only fair or even poor. Clearfield and Clearfield Plus herbicides can be applied over the top of CLPlus sunflowers up to 10 weeks after crop emergence, and rainfall is not required to activate the herbicide. These herbicides are used at relatively low rates of application and control a very wide spectrum of dicot and grass weed species (Pfenning et al, 2008). Both Clearfield and Clearfield Plus herbicides are also the only herbicides that deliver effective chemical control of an extremely damaging and difficult-to-control parasitic weed, *Orobanche cumana*, which feeds off the roots of the host sunflower plant, and can reduce sunflower grain yield by 50 to- 80% depending on the level of infestation. The use of these herbicides complement the efforts being carried out at the research and breeding levels to introduce new genetic sources of *Orobanche* tolerance into cultivated sunflowers (Labrousse et al, 2011).

Different adjuvants, such as MSO (Methylated Seed Oil) or BASF's proprietary adjuvant, DASH® that enhance the activity of Clearfield herbicides, were field tested from 2007 to 2011 in different market regions. Due to the higher imidazolinone tolerance of the CLPlus trait the use of a stronger adjuvant was well tolerated and provided better weed control options. The combination of a stronger adjuvant and a better imidazolinone formulation in Clearfield Plus will provide growers with a more flexible and reliable weed control system in sunflower as well as the possibility of relaxed re-cropping restrictions (Pfenning et al, 2012).

Another advantage of the higher tolerance level of CLPlus is a better stability of imidazolinone tolerance to cope with the unpredictable portion of the environmental variation (pre- and post- application weather conditions for example). This greater stability was shown to provide a better reliability of the CLPlus technology than the Imisun trait across different imidazolinone molecules and rates (Sala et al., 2012b). Since sunflowers are often grown in more marginal areas, better reliability of a crop's performance after herbicide application and improved weed control are valuable advantages to a grower. As well, better weed control and greater stability of the herbicide tolerance trait across environments lead to improved yield in CLPlus hybrids. What still is under investigation is whether the CLPlus trait, in the absence of weed pressure, out-yields the ImiSun trait. Data from a limited set of isogenic hybrids indicate that CLPlus hybrids yield the same as their conventional isogenic counterparts, yet yield significantly higher than their ImiSun isogenic counterparts. A strong trend for larger grain size in CLPlus hybrids was also observed (Weston et al, 2012). More data from isogenic hybrids from different genetic backgrounds need to be evaluated in multiyear agronomic field trials to help substantiate this data.

Sunflower oil is still the fourth largest vegetable oil in the world, after soybean, palm and canola. Many countries, including Argentina, pay an oil premium to growers who deliver above average levels of grain oil yield. Preliminary studies on isogenic hybrids have shown a 1% - 2% significant increase in oil yield in CLPlus hybrids over their ImiSun isogenic counterparts (Weston et al, 2012). This oil boost is believed to be associated with the fact that the *CLHA-Plus* mutation has no linked wild sunflower sequences, as is the case for the *Ahasl1-1* mutation in ImiSun (Trucillo et al, 2010). Similar to the preliminary yield observations, the increased levels of grain oil in CLPlus need to be substantiated in different genetic backgrounds.

CONCLUSIONS:

The Clearfield Plus system provides ease of use for both the variety developer and the grower. The introduction of this new production system will lead to improved weed control, better yields and higher grain oil content in sunflowers with the greatest gains experienced in marginal growing areas. It is anticipated that this innovation will open the door to more investment in herbicide tolerance systems; investment which will develop more options for weed resistance management and make sunflowers more competitive longterm. The Clearfield Plus production system is scheduled for registration in countries around the globe as early as 2012 and will be available in North and South America, Russia, South Africa, Eastern Europe and Western Europe. The trait will be developed and sold in partnership with many seed companies worldwide.

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